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FUNCTIONAL AND MOLECULAR PROPERTIES OF DIHYDROPYRIDINE-BINDING PROTEINS IN RABBIT SKELETAL MUSCLE TRANSVERSE TUBULES

BY

MANJUNATHA B. BHAT

©

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To

My Mother

And Family

ABSTRACT

A rapid filtration technique was developed to investigate the kinetics of depolarization-induced $^{45}Ca^{2+}$ flux mediated by voltage-dependent Ca^{2+} channels (VDCCs) in transverse tubule membrane vesicles isolated from rabbit skeletal muscle. Membrane vesicles which are inside-out in orientation were loaded with $^{45}Ca^{2+}$ and repolarized to an estimated -86 mV by establishing potassium diffusion gradients in the presence of a potassium ionophore, valinomycin. Upon subsequent depolarization, the greatest amount of specific $^{45}Ca^{2+}$ efflux was measured within 300 msec of initiation of depolarization.

At equimolar (1 mM) concentration, the inorganic Ca^{2+} channel blocker, La^{3+} completely inhibited the depolarization-induced $^{45}Ca^{2+}$ flux response, whereas Cd^{2+} was partially effective in inhibiting the flux response. The 1,4-dihydropyridine (DHP) agonist (±)Bay K8644 (0.1 μM) significantly stimulated $^{45}Ca^{2+}$ efflux induced by depolarization as well as reduced the amount of $^{45}Ca^{2+}$ retained by the vesicles under control conditions.

Extravesicular (i.e. intracellular) Ca^{2+} was found to inhibit depolarization-induced $^{45}Ca^{2+}$ efflux in a concentration-dependent manner with an IC_{50} value of 0.7 μM, suggesting Ca^{2+}-dependent inactivation of VDCCs in skeletal muscle. Furthermore, the amount of $^{45}Ca^{2+}$ efflux measured by depolarization was increased when the repolarizing potential was more negative suggesting the presence of a

voltage-dependent channel inactivation process in skeletal muscle. The DHP antagonist, nifedipine (10 μM) was found to show voltage-dependent blocking effect on the $^{45}Ca^{2+}$ flux response. The inhibitory effect of nifedipine was greater when the $^{45}Ca^{2+}$ flux response was elicited from a less negative (-30 mV) repolarizing potential. However, the high affinity binding of the DHP ligand, [^{3}H]PN200-110 to isolated transverse tubule membrane vesicles was found to be voltage-independent.

At micromolar concentrations, the fluorescent DHP antagonist, amlodipine was found to accelerate the rate of dissociation of [^{3}H]PN200-110 from its binding sites in the transverse tubule membranes. The fluorescence of amlodipine increases upon its binding to transverse tubule membranes, and this increase in fluorescence was found to be DHP-specific and saturable with an apparent dissociation constant of approximately 5 μM.

Based on the above observations, it is concluded that isolated transverse tubule membranes from rabbit skeletal muscle can be used to study the voltage-dependent Ca^{2+} channel activity of dihydropyridine-binding proteins (DHPBPs) in their native membrane environment. Furthermore, it is concluded that DHP drugs modulate VDCC activity in a voltage-dependent manner. This channel-modulating effect of DHP drugs may involve their binding to low affinity binding sites located either on or near high affinity binding sites on the Ca^{2+} channel protein complex.

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ABBREVIATIONS

α	alpha
ACS	aqueous counting scintillant
ATP	adenosine triphosphate
β	beta
Ba	barium
BT	Benzothiazepine
Ca	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Cd	cadmium
cDNA	complementary DNA
Ci	curie
δ	delta
DHP	1,4-dihydropyridine
DHPBP	dihydropyridine-binding protein
diS-C_3-(5)	3,3'-dipropyl-2,2'-thiadicarbocyanine iodide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
e-c	excitation-contraction

EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
E_m	equilibrium membrane potential
γ	gamma
g	gram
G protein	guanine nucleotide binding protein
G_s	GTP binding protein stimulatory to adenylate cyclase
Glu	glutamic acid
GTP	guanosine triphosphate
GTP-γ-S	guanosine-5'-[3-thio]-triphosphate
h	hour
HCl	hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA	high voltage activated
K	potassium
K_d	dissociation constant
kD	kilo dalton
l	litre
La	lanthanum
LVA	low voltage activated
m	milli
M	mole/l

mg	milligram
min	minute(s)
ml	millilitre
n	nano
Ni	nickel
nm	nanometre
p	pico
PA	phenylalkylamine
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
pS	pico Siemens
rpm	rotations per minute
sec	second(s)
Sr	strontium
SR	sarcoplasmic reticulum
t-tubule	transverse tubule
Tris	Tris(hydroxymethyl)aminomethane
Trp	tryptophan
μ	micro
V	volt
VDCC	voltage-dependent calcium channel
w/v	weight/volume

1. INTRODUCTION

1.1 CALCIUM CHANNELS

1.1.1 Introduction

Calcium (Ca^{2+}) ions play an essential role in many key biological processes including the maintenance of cellular organization, cell multiplication and motility, bone formation and the coupling of stimuli to their mechanical, secretory or metabolic responses. It is now widely accepted that Ca^{2+} plays a major role as a second messenger in excitation-contraction (e-c) coupling in cardiac, skeletal, and smooth muscle, and in the regulation of neurotransmitter and hormone secretion from many different cellular systems (Rasmussen and Barret, 1984; Rubin *et al.*, 1985). Typically the concentration of Ca^{2+} in the extracellular fluid is 1-10 mM whereas the intracellular concentration of free Ca^{2+} ranges from 0.1 to 10 μM, depending on the state of the cell. The low resting intracellular level of Ca^{2+} (about 0.1 μM) increases by one to two orders of magnitude in response to either chemical or electrical stimulation. Several different types of Ca^{2+} transport systems that are present both on the cell surface membrane and on the intracellular organelles (such as endoplasmic/sarcoplasmic reticulum) operate to maintain the low concentration of intracellular free Ca^{2+} (Carafoli, 1987).

The major entry pathway for Ca^{2+} in many cell types is via Ca^{2+} channels that are anchored to the plasma membrane. These channels are structures consisting of

a functional pore capable of transferring millions of permeant cations (i.e. Ca^{2+} and Ba^{2+}) per second across the cell membrane. The opening of these channels enables Ca^{2+} to move passively across the membrane down its electrochemical gradient into the cell.

1.1.2 Types of Ca^{2+} channels

Different types of Ca^{2+} channels exist and they are characterized by differences in the mechanisms of their opening and closing (gating). Some channels are voltage-dependent and are gated primarily by changes in membrane potential (Catterall, 1988; Hosey and Lazdunski, 1988; Bean, 1989; Tsien *et al* ., 1991; Miller, 1992; Dunn *et al* ., 1993). However, they may also be regulated by receptors that are directly coupled to the channels by means of GTP-binding proteins or through the action of diffusible second messengers and related phosphorylation systems. Within this category, subclasses of Ca^{2+} channels have been identified based on biophysical and pharmacological characteristics. These voltage-dependent Ca^{2+} channels (VDCCs) will be discussed in detail in later sections.

Another category of Ca^{2+} channels includes channels that are operated through receptor-dependent mechanisms. These channels are closely linked to membrane receptors and are activated by agonist binding to the receptor to stimulate the influx of Ca^{2+} in the absence of any changes in membrane potential (Bolton,

1979; Meisheri *et al.*, 1981). Ca^{2+} influx through receptor-operated Ca^{2+} channels may, however, depolarize the cell membrane and indirectly activate VDCCs (Siegel and Adler, 1985). Examples of receptor-operated Ca^{2+} channels include those in smooth muscle that are opened by activation of ATP receptors (Benham and Tsien, 1987) and parathyroid hormone-activated Ca^{2+} channels in osteosarcoma cells (Yamaguchi *et al.*, 1987). Although the receptor-operated Ca^{2+} channels play vital roles in the function of smooth muscle and secretory cells, they have received less scrutiny than the VDCCs, and the lack of experimental data characterizing these channels has limited our knowledge of their specific functional properties (Hurwitz, 1986).

Other types of Ca^{2+} channels include leak channels which mediate Ca^{2+} influx as a consequence of the large electrochemical gradient which tends to drive Ca^{2+} into the cell (Ress and Flaim, 1982). Although this is referred to as a "channel" mechanism for reasons of convenience, it is unlikely to occur via a gated channel of classical structure. Increased leak channel activity has been suggested to be the cause of elevated resting free intracellular Ca^{2+} levels observed in dystrophic mouse (*mdx*) skeletal muscle fibres and myotubes and human Duchenne muscular dystrophy myotubes (Fong *et al.*, 1990; Turner *et al.*, 1991).

In vascular smooth muscle, Ca^{2+} may also enter cells through stretch-sensitive channels which again are not classical gated channels. An increased Ca^{2+} leak

through mechanically perturbed cell membranes may be explained by such a mechanism (Schramm and Towart, 1985).

1.1.3 Voltage-Dependent Ca^{2+} Channels (VDCCs)

Voltage-dependent Ca^{2+} channels (VDCCs) are present not only in excitable cells such as muscle cells (smooth, cardiac and skeletal), nerve cells (central and peripheral), and many secretory cells, but also in many non-excitable cells including glial cells, myeloma cells, osteoblasts and fibroblasts (Tsien *et al.*, 1988; Barren *et al.*, 1988; Fukushima and Hagiwara, 1988; Chesnoy-Marchais and Fritsch, 1988; Chen *et al.*, 1988; Hess, 1990). Based on their electrophysiological characteristics and specific drug and toxin sensitivity, three types (L, N and T) of VDCC currents were originally identified in chick dorsal root ganglion (DRG) neurons. A fourth (P) type has recently been described in cerebellar Purkinje cells (Nowycky *et al.*, 1985; Tsien *et al.*, 1988; Llinas *et al.*, 1989; Spedding and Paoletti, 1992).

L-type Ca^{2+} channels, named for the long-lasting openings and the largest conductance (approximately 20-25 pS in 100 mM Ba^{2+}) seen by single channel recording, are the best characterized VDCCs, and are present in virtually all excitable and many non-excitable tissues (Spedding and Paoletti, 1992). They are described as high voltage activated (HVA), and are sensitive to 1,4 dihydropyridine (DHP) compounds. L-type channels generate a slowly inactivating and rapidly

deactivating current that is activated by large depolarizations from relatively depolarized holding potentials. These channels exhibit three gating characteristics: mode 1 is characterized by brief openings (with mean open times of approximately 1 ms), mode 2 occurs less frequently but with much longer openings, and in mode 0 the channels do not open at all (Hess *et al.*, 1984).

Ca^{2+} currents through L-type channels display both Ca^{2+}-dependent and voltage-dependent inactivation (Chad, 1989; Pelzer *et al.*, 1990). An increase in intracellular Ca^{2+} concentration results in inactivation of the Ca^{2+} current. The rate of Ca^{2+} current inactivation thus increases with increasing Ca^{2+} currents and slower inactivation is observed when external Ca^{2+} is replaced with Ba^{2+} or Sr^{2+} as charge carrier, or when the Ca^{2+} entering through the channel is buffered with intracellular EGTA (Lee *et al.*, 1985). Ca^{2+} channel currents recorded using either Ba^{2+} or Sr^{2+} (to avoid Ca^{2+} dependent inactivation) undergo inactivation during a step depolarization indicating the presence of a voltage-dependent mechanism (Lee *et al.*, 1985). Inactivation is also produced by prepulses that do not activate measurable Ca^{2+} currents, further suggesting a voltage-dependent inactivation of the Ca^{2+} current (Campbell *et al.*, 1988a).

L-type channels are sensitive to either block or activation by 1,4 dihydropyridine derivatives and this feature is used to distinguish L-type channels from T-, N-, and P-type Ca^{2+} channels (Tsien *et al.*, 1991). L-type channels can also

be distinguished from T-type channels by their sensitivity to holding potentials. At fairly depolarized holding potentials (>-40 mV), all T-channels are generally inactivated whereas L-channels are not. L-type channels are also more sensitive to block by Cd^{2+} and less sensitive to Ni^{2+} than are T-channels (Miller and Fox, 1990). Another characteristic of L-type Ca^{2+} channels is the requirement of the presence of an intracellular phosphorylating system for the maintenance of channel activity (Armstrong and Eckert, 1985; 1987; Levitan and Kramer, 1990). For example, in whole-cell or excised patch recordings, the L- channel currents gradually 'rundown' suggesting that some cytoplasmic factors are responsible for the maintenance of channel activity (Chad and Eckert, 1986). Exogenously added cAMP-dependent protein kinase was able to maintain active L channel function in GH_3 clonal pituitary cells (Armstrong and Eckert, 1987), whereas perfusion of neurons with the Ca^{2+}-dependent phosphatase, calcineurin, abolishes the Ca^{2+} channel activity (Chad and Eckert, 1986).

N-type Ca^{2+} channels (Nowycky *et al.*, 1985) have been found exclusively in neuronal cells where they have been suggested to play a role in neurotransmitter release (Hirning *et al.*, 1988). N-type channels are also "high voltage activated", but pharmacologically differ from L-channels in being resistant to dihydropyridines and in some species, being sensitive to an ω-toxin from *Conus geographus* (ω-CgTx GVIA) (McCleskey *et al.*, 1987). N-type channels may be modulated by receptor-linked second messenger systems such as G-proteins (Kongsamut *et al.*, 1989). The N-

channels are also distinguished from L-type channels by having a smaller single channel conductance (approximately 13 pS in 110 mM Ba^{2+}) and by their rapid inactivation (Fox *et al.*, 1987).

T-type (for tiny or transient) Ca^{2+} channels, are low voltage activated (LVA) Ca^{2+} channels, and are different from L- and N-type channels in that they are activated by smaller depolarizations from relatively negative holding potentials. T-type channel current is activated at about -70 mV and reaches its peak value at about -40 mV. T-type channel current is characterized by a small single channel conductance of only 5-10 pS in 100 mM Ba^{2+}, rapid voltage-dependent inactivation during a prolonged pulse (Nowycky *et al.*, 1985; Fox *et al.*, 1987; Bean, 1989) and slow deactivation (Carbone and Swandulla, 1989). Because T-channels are activated at potentials close to resting membrane potentials, these channels have been implicated in spontaneous membrane potential fluctuations and burst firing in neurones (White *et al.*, 1989). T channels have also been suggested to play a role in development, and in long-term potentiation in the mossy fibre pathway (Cobb *et al.*, 1989; Kamiya, 1989). The pharmacology of the T-type Ca^{2+} channel has not been well developed, mainly because of the lack of selective ligands. However, the pyrazine diuretic, amiloride (Tang *et al.*, 1988), and high molecular weight alcohols such as octanol, nonanol and decanol (Llinas and Yarom, 1986) have been shown to exert a selective blocking action on these channels.

Recently, a third high-voltage activated Ca^{2+} channel was described and termed P-type since it was first found in Purkinje cells of the cerebellum (Llinas *et al.*, 1989). These channels activate at potentials less negative than -50 mV and show very slow inactivation. They are very susceptible to block by both peptide (agatoxins) and polyamine (FTX) toxins derived from American funnel-web spider (*Agelenopsis aperta*) venom. They differ from other high-voltage activated Ca^{2+} channels in being insensitive to either 1,4-dihydropyridines or ω-conotoxins (Tsien *et al.*, 1991) and also at the level of single channel properties (10-12 pS in 80 mM Ba^{2+} and 6-8 pS in 100 mM Ca^{2+}) (Llinas *et al.*, 1989). Although the P-type Ca^{2+} channel appears to be the most widely distributed Ca^{2+} channel in the mammalian CNS (Hillman *et al.*, 1991; Bertolino and Llinas, 1992), Ca^{2+} currents with similar kinetics and pharmacology have also been found in peripheral neurones (Plummer *et al.*, 1989; Regan *et al.*, 1991).

1.2 PHARMACOLOGY OF L-TYPE VDCCs

A large number of synthetic and naturally occurring compounds have been shown to modulate the activity of VDCCs. Most of the major drug classes described so far appear to be relatively specific for L-type Ca^{2+} channels, although drugs and toxins that act relatively specifically on other types of Ca^{2+} channels have been recently identified. Because of their wide-spread therapeutic importance in treating cardiovascular diseases, drugs acting on L-type Ca^{2+} channels have been the subject

of major attention in the past decade (Janis and Triggle, 1987; Triggle and Rampe, 1988). Further, some of the agents have served as biochemical probes in elucidating the molecular properties of VDCCs (Hosey and Lazdunski, 1988). A variety of terms, including Ca^{2+} antagonist, Ca^{2+} channel blocker, and slow channel blocker, have been used to identify a structurally heterogeneous group of agents that specifically bind to VDCCs and inhibit the movement of Ca^{2+} through them (Fleckenstein, 1988). However, with the introduction of 1,4-dihydropyridine analogues (such as Bay K8644 and CGP 28392) that function as Ca^{2+} channel activators, a more generic title "Ca^{2+} channel ligands" is used to recognize drugs that modify the function of VDCCs (Schramm *et al.*, 1983).

Although Ca^{2+} channel ligands have been classified based on physiological, pharmacological, and therapeutic criteria, popular classification is based on their basic chemical structure, according to which there are five classes of drugs (Glossmann and Striessnig, 1990): (i) 1,4-dihydropyridines (DHPs) such as nifedipine and nitrendipine, (ii) phenylalkylamines such as verapamil and D-600, (iii) benzothiazepines such as diltiazem, (iv) diphenylbutylpiperidines such as fluspirilene, and (v) benzothiazinones such as HOE-166. It is currently believed that drugs from each of these five classes recognize a distinct binding domain on the α_1 subunit of the L-type Ca^{2+} channel, and that these receptor binding domains allosterically interact with each other. Some drugs belonging to the 1,4-DHP class (e.g. Bay K8644, SDZ 202-791) exhibit a Ca^{2+} channel activating effect. Furthermore, different optical

isomers of DHPs have been shown to produce opposing effects on Ca^{2+} channel activity (Hof *et al.*, 1985; Franckowiak *et al.*, 1988). For example, (+)SDZ 202-791 acts as a channel agonist whereas its (-) isomer acts as an antagonist. Thus, Ca^{2+} channel ligands have complex pharmacological actions.

Different Ca^{2+} channel ligands show selectivity towards specific tissues for their action. For example, drugs such as nifedipine have a relatively selective action on vascular tissues compared to cardiac tissues, whereas verapamil-like drugs are very effective on cardiac tissues. This selectivity of action may be attributed to the mechanism of action of these drugs. Thus, verapamil-like drugs block VDCCs better in situations where the channels open and close frequently (frequency-dependent block) (Lee and Tsien, 1983). On the other hand, the effects of DHPs are controlled by changes in the membrane potential, their potency being increased at a depolarized membrane potential because of the state-dependent binding of drugs to the channels (Bean, 1984; Sanguinetti and Kass, 1984). In general the agonist effect of a compound is favoured by a hyperpolarized membrane potential whereas the antagonist effect is seen at depolarized potentials (Kass, 1987; Kamp *et al.*, 1989).

1.3 SKELETAL MUSCLE DHP-SENSITIVE VDCCs

The presence of VDCCs in skeletal muscle was revealed by the demonstration of slow Ca^{2+} action potentials in frog skeletal muscle fibres (Beaty and Stefani, 1976;

Chiarandini and Stefani, 1983; Kerr and Sperelakis, 1983). Such action potentials are sensitive to blockade by Ca^{2+} channel blockers including verapamil, D600 and nifedipine. Voltage-clamp studies have demonstrated the presence of a slow inward current that is carried by Ca^{2+} (or Ba^{2+}) and is abolished in the absence of external Ca^{2+}, or in the presence of Co^{2+}, Cd^{2+}, or D600 (Stanfield, 1977; Sanchez and Stefani, 1978; Almers and Palade, 1981; Donaldson and Beam, 1983). Ca^{2+} currents can be detected with depolarization close to -40 mV which is near the mechanical threshold of muscle cells (Hodgkin and Horowicz, 1960; Stefani and Chiarandini, 1982; Sakar *et al.*, 1986; Best *et al.*, 1991). However, the Ca^{2+} channels in skeletal muscle have activation kinetics that are much slower (with time to peak conductance of about 100-200 msec) than the time course of a twitch contraction (about 10 msec).

The slow Ca^{2+} channels in the skeletal muscle have been shown to be located in the transverse tubule membrane system. Muscle fibres in which transverse tubules are electrically uncoupled from the surface membrane by glycerol treatment exhibit Ca^{2+} currents that are substantially reduced from those of control fibres (Nicola Siri *et al.*, 1980). Such fibres, however, propagate action potentials with normal sodium and potassium currents. Furthermore, in experiments with cut fibre preparations, the rate of decline of the Ca^{2+} current was increased following depletion of Ca^{2+} in the transverse tubule lumen (Almers *et al.*, 1981). Further evidence for the transverse tubular localization of VDCCs was obtained from the finding that the density of binding proteins for the DHP class of Ca^{2+} channel ligands is several orders of

magnitude higher than in any other tissues (Fosset *et al.*, 1983).

Like activation, the inactivation of slow L-type Ca^{2+} current is also much slower in skeletal muscle fibres (Pelzer *et al.*, 1990) than in other tissues and occurs over several seconds. This long inactivation process was attributed to the time-dependent depletion of Ca^{2+} in the transverse tubules (Almers *et al.*, 1981). It was also observed that in embryonic skeletal muscle myotubes which have a poorly developed transverse tubule system, slow Ca^{2+} currents show no inactivation during activating, depolarizing pulses whereas slow inactivation became apparent in similar preparations from adult animals with an established transverse tubule system (Beam and Knudson, 1988). In line with these findings, purified skeletal muscle Ca^{2+} channels reconstituted in lipid bilayers do not show inactivation (Flockerzi *et al.*, 1986; Pelzer *et al.*, 1989).

There is also evidence for the presence of voltage-dependent inactivation of slow Ca^{2+} channels in skeletal muscle (Sanchez and Stefani, 1983; Cota *et al.*, 1984; Beaty *et al.*, 1987; Beam and Knudson, 1988). In paired-pulse experiments with intact skeletal muscle fibres, the amount of inactivation seen is independent of the Ca^{2+} current amplitude seen with the conditioning prepulse (Sanchez and Stefani, 1983; Cota *et al.*, 1984).

In addition to the slow Ca^{2+} current, a fast activating Ca^{2+} current has also

been identified in skeletal muscle fibres (Cota and Stefani, 1986). This low threshold (-60 mV), rapidly activating current is insensitive to dihydropyridine-block, a characteristic feature of the T-type Ca^{2+} current described in other tissues. However, this current does not show appreciable inactivation. The fast kinetic properties of this current is suggestive of its possible activation during a single action potential and thus its possible contribution to the Ca^{2+} influx measured during a twitch contraction (Bianchi and Shanes, 1959; Curtis, 1966).

1.4 MOLECULAR PROPERTIES OF SKELETAL MUSCLE DHP-SENSITIVE VDCCs

1.4.1 Subunit Structure

The molecular structure of the voltage-dependent, DHP-sensitive Ca^{2+} channel is being studied using pharmacological, biochemical, immunological, and molecular biological approaches. The availability of radiolabelled DHP ligands has greatly aided in the isolation and characterization of these channel proteins. Although DHP-sensitive Ca^{2+} channels are present in virtually all excitable and many non-excitable cells, rabbit skeletal muscle has been shown to be the richest source of DHP binding proteins (Fosset *et al.*, 1983; Caterall, 1991). Since the localization of DHP binding proteins in the transverse tubule system is consistent with the known localization of VDCCs in this tissue, skeletal muscle membranes have been used as

the source for purification and characterization of putative L-type Ca^{2+} channels (Sanchez and Stefani, 1978; Fosset *et al.*, 1983; Glossmann *et al.*, 1983).

Using conventional receptor purification procedures involving detergent solubilization and affinity chromatography, the DHP binding protein has been purified from skeletal muscle by several laboratories (Curtis and Caterall, 1984; Borsotto *et al.*, 1984). Purified DHP binding proteins have been shown to exhibit Ca^{2+} channel activity when reconstituted into either phospholipid vesicles or lipid bilayers (Curtis and Catterall, 1986; Smith *et al.*, 1988). It is now widely accepted that the DHP binding protein is a hetero-oligomeric complex consisting of five distinct subunits: two high molecular weight subunits (α_1 and α_2 with 170 kDa and 140 kDa, respectively) and three subunits with low molecular weights [β (55 kDa), γ (32 kDa) and δ (30 kDa)]. Though these five subunits have a usual stoichiometry of 1:1:1:1:1 (Catterall *et al.*, 1988), variability in this pattern may be observed during the course of muscle development (Morton and Froehner, 1989; Varadi *et al.*, 1989). The α_2 and δ subunits are linked by disulphide bond and are encoded by the same gene (DeJongh *et al.*, 1990; Jay *et al.*, 1991). The β subunit which is hydrophillic and non-glycosylated, contains many consensus sequences for phosphorylation sites. The γ subunit is heavily glycosylated and has been suggested to contain membrane-spanning regions.

The complementary DNAs for all the five subunits of skeletal muscle DHP

binding protein have been purified and sequenced (Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989; DeJongh *et al.*, 1990; Jay *et al.*, 1990). A structural model of the DHP-sensitive Ca^{2+} channel, based on those described earlier (Catterall, 1988; Campbell *et al.*, 1988b; Dascal, 1990) is shown in figure 1. Detailed structures and functional role(s) of each of the five subunits are discussed below.

The α_1 subunit is the main functional subunit of the DHP binding protein since this subunit alone is capable of forming a functional Ca^{2+} channel when expressed in murine L cell lines (Perez-Reyes *et al.*, 1989) or when reconstituted in lipid bilayers (Pelzer *et al.*, 1989). Photoaffinity labelling studies have demonstrated that the α_1 subunit also carries binding sites for classical Ca^{2+} channel ligands of dihydropyridine and other classes (Striessnig *et al.*, 1986; Striessnig *et al.*, 1987; Sieber *et al.*, 1987; Naito *et al.*, 1989). The α_1 subunit also contains several phosphorylation sites, which *in vitro* may be phosphorylated by cAMP-dependent protein kinase (kinase A), protein kinase C, Ca^{2+}-calmodulin kinase, casein kinase II and cGMP kinase (Röhrkasten *et al.*, 1988; Jahn *et al.*, 1988). The primary amino acid sequence of the α_1 subunit deduced from the cDNA sequence is 1873 amino acids long and predicts the molecular mass of this subunit to be about 210 kDa (Tanabe *et al.*,1987). This sequence displays about 30% homology to the α_1 subunit of the voltage-dependent sodium channel (Tanabe *et al.*, 1987) and a K^+ channel (I_A) cloned from *Drosophila* (Schwartz *et al.*, 1988) suggesting that an evolutionary relationship exists between these voltage-gated channels (Miller and Fox, 1990). The primary sequence

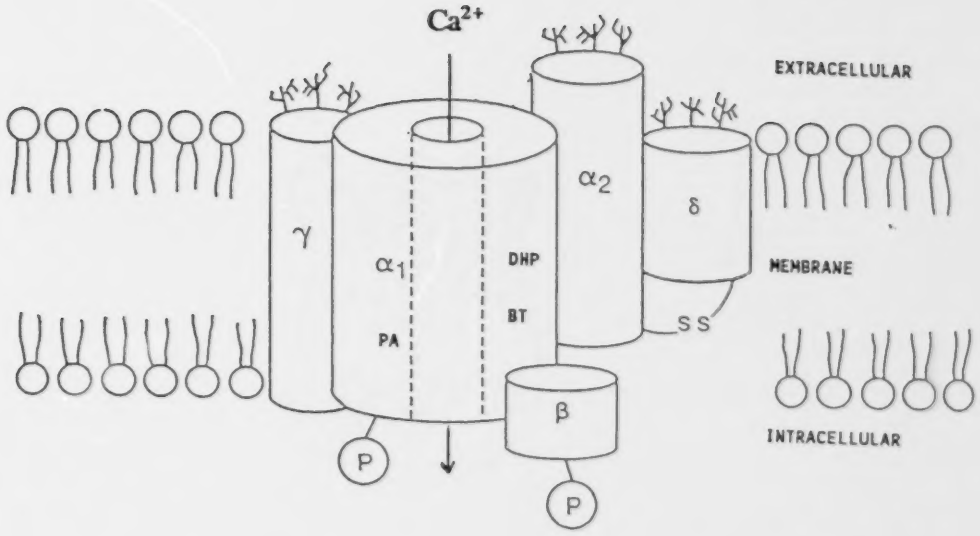


Figure 1. Proposed structural model of the skeletal muscle DHP-sensitive Ca^{2+} channel complex (modified from Dunn *et al.*, 1993).

DHP= Dihydropyridine binding site; PA= Phenylalkylamine binding site; BT= Benzothiazepine binding site; S-S= Disulphide bond; P= Phosphorylation sites; = Glycosylation sites.

of the α_1 subunit contains four homologous domains, each of which is predicted to contain six hydrophobic regions with potential α-helical structures that span the membrane. The fourth transmembrane helix (S4) of each of the four domains contains positively charged amino acids (arginine or lysine) at every 3rd or 4th residue and is thought to constitute the voltage-sensing region of the channel protein (Catterall, 1988; Miller, 1992).

1.4.2 Homologous VDCCs

Following the characterization of the α_1 subunit of skeletal muscle L-type VDCC, a variety of other homologous channel proteins has been identified by using molecular biological techniques. The α_1 subunit of the cardiac Ca^{2+} channel shows 66% homology to the skeletal muscle α_1 subunit, and forms a functional cardiac muscle VDCC with fast activation kinetics when expressed in *Xenopus* oocytes (Mikami *et al.*, 1989). Snutch *et al* (1990) and Perez-Reyes *et al* (1990) independently identified four distinct classes of cDNA clones encoding Ca^{2+} channel α_1 subunit from rat brain. These clones were termed rbA or 4, rbB or 5, rbC or 2, and rbD or 3, with letters used by Snutch *et al* (1990) and numbers by Perez-Reyes *et al* (1990). The full length cDNAs corresponding to class C clone isolated from rabbit lung (Biel *et al.*, 1990), rat aorta (Koch *et al.*, 1990) and rat brain (Snutch *et al.*, 1991) show ~97% sequence identity to the cardiac α_1 subunit. There is a high degree of homology between classes and A & B and between classes C & D (Tsien *et al.*,

1991). However, the putative voltage sensor domain (S4) differs between A, B and C, D suggesting potential differences in electrophysiological properties (Spedding and Paoletti, 1992).

Recently, full length cDNAs related to class D that encode DHP-sensitive Ca^{2+} channels have been isolated from a variety of tissues (Miller, 1992; Dunn *et al.*, 1993). Full length A class clones termed BI (Mori *et al.*, 1991) and rbA-I (Staar *et al.*, 1991) have been obtained from rabbit and rat brains, respectively. When expressed in *Xenopus* oocytes, the BI channels show partial inactivation and are not sensitive to 1,4-dihydropyridine derivatives and ω-conotoxin, but are sensitive to block by crude venom from American funnel web spider (Mori *et al.*, 1991) suggesting that these may be 'P' type channels. However, there are substantial differences between BI (class A) channels and P-type channels (Tsien *et al.*, 1991). Based on the pattern of sequence homology, it has been suggested that all Ca^{2+} channels evolved from a single ancestral gene, and the functional diversity among these channels results not only through gene duplication, but also through alternative splicing at different key regions (Tsien *et al.*, 1991).

1.4.3 Structure-Function Relationship of DHP-Sensitive VDCCs

Although it is difficult to express the skeletal muscle α_1 subunit in transient expression systems such as *Xenopus* oocytes, this protein has been expressed in

murine L cell lines (Perez-Reyes *et al.*, 1989). This protein forms a DHP-sensitive Ca^{2+} channel in the absence of the other subunits (α_2/δ, β and γ). However, the gating characteristics of the current in this expression system are different from native skeletal muscle Ca^{2+} channels suggesting the possible requirement for either the 'regulatory' subunits or the normal transverse tubule environment for normal functioning of the channel (Perez-Reyes *et al.*, 1989; Lacerda *et al.*, 1991; also see section 1.4.4 below).

The functional importance of the α_1 subunit for Ca^{2+} channel activity has been further studied using molecular biological approaches. Cultured skeletal muscle cells from mice with muscular dysgenesis lack the normal α_1 subunit (Knudson *et al.*, 1989). These cells do not exhibit electrically stimulated e-c coupling and lack significant DHP-sensitive Ca^{2+} channel activity (Pincon-Raymond *et al.*, 1985; Beam *et al.*, 1986). Injection of cDNA encoding the α_1 subunit into the nucleus of such deficient myotubes results in the restoration of both slow Ca^{2+} channel activity and skeletal muscle type e-c coupling (Tanabe *et al.*, 1988). On the other hand, expression of the cardiac muscle α_1 subunit in such myotubes produces cardiac-type Ca^{2+} channel activity with fast activation kinetics and a contractile response typical of cardiac muscle.

Tanabe et al (1990; 1991), used chimeric Ca^{2+} channels to study the structural determinants of the α_1 subunit that confer either cardiac or skeletal muscle type

channel activity and e-c coupling. E-c coupling in cardiac muscle is dependent on extracellular Ca^{2+} whereas the initiation of contraction in skeletal muscle is independent of external Ca^{2+}. In a chimeric channel expressed in dysgenic myotubes using cDNA encoding the cardiac α_1 subunit, replacement of the intracellular loop connecting the domains 2 and 3 with the skeletal muscle counterpart resulted in a cell that showed skeletal muscle-like e-c coupling, but cardiac-like Ca^{2+} current (fast activating) (Tanabe *et al.*, 1990). On the other hand, changing only the first homologous domain to that of the skeletal muscle type switched the nature of the Ca^{2+} current from cardiac to skeletal muscle type (slowly activating) (Tanabe *et al.*, 1991).

1.4.4 Functional Role of the Auxiliary Subunits of DHP-Sensitive VDCCs

Recently, there have been many studies directed at examining the functional significance of the other subunits (α_2/δ, β and γ) that copurify with the α_1 subunit. Cloning and sequencing of the disulphide-linked α_2/δ subunit has indicated that it is highly glycosylated (18 potential glycosylation sites) and that it contains two potential cAMP-dependent phosphorylation sites (Ellis *et al.*, 1988). Proteins with primary sequence identical or closely related to the skeletal muscle α_2/δ subunit have been identified in other tissues including neurons, cardiac muscle and smooth muscle (Ellis *et al.*, 1988; Williams *et al.*, 1992). Antibodies raised against the skeletal muscle α_2/δ subunit could immunoprecipitate the DHP binding protein complex from both

skeletal muscle and brain, whereas less than 10% of the brain high affinity ω-CgTx GVIA binding sites (presumably representing the N-type Ca^{2+} channels; see above) were coprecipitated suggesting that α_2/δ subunits are associated with at least some of the brain L-type Ca^{2+} channels (Ahlijanian *et al.*, 1990; 1991). Both the α_2 (N-terminal) and δ (C-terminal) portions of the protein contain hydrophobic sequences and it has been suggested that the δ portion anchors the α_2 portion to the plasma membrane (Jay *et al.*, 1991).

The α_2/δ subunits have also been shown to affect the Ca^{2+} channel activity of the α_1 subunit. Co-expression of the skeletal muscle α_2/δ subunits substantially potentiated the Ca^{2+} current mediated by the cardiac α_1 subunit expressed in *Xenopus* oocytes (Mikami *et al.*, 1989; Singer *et al.*, 1991). In recombinant studies using the skeletal muscle α_1 subunit, addition of α_2/δ subunit enhanced the rate of voltage-dependent Ca^{2+} influx through L-channels (Gutierrez *et al.*, 1991). Further, the co-expression of the α_2/δ subunit in murine L cells with the skeletal muscle α_1 subunit resulted in an increase in the density of DHP binding sites without any change in binding affinity (Varadi *et al.*, 1991).

The β subunit of the DHP-sensitive Ca^{2+} channel is thought to represent an extrinsic membrane protein associated with the cytoplasmic portion of the α_1 subunit (Miller, 1992). Multiple forms of the β subunit have been shown to exist in different tissues (Perez-Reyes *et al.*, 1992; Hullin *et al.*, 1992; Castellano *et al.*, 1993; Collin *et*

al., 1993). The β subunit copurifies with the α_1 subunit from both skeletal muscle and brain (Ahlijanian *et al.*, 1990). Further, antisera directed against the skeletal muscle β subunit coprecipitate both DHP binding and ω-conotoxin binding sites from brain (Sakamoto and Campbell, 1991). Functionally, co-expression of the α_1 and β subunits results in a ten-fold increase in DHP binding sites with no change in the binding affinity, and the activation and inactivation kinetics of the Ca^{2+} current were accelerated by this co-expression (Varadi *et al.*, 1991). Further, co-expression of the skeletal muscle β subunit with the skeletal muscle α_1 subunit in murine L cells resulted in Ca^{2+} channel currents with normal activation kinetics suggesting a functional role of β subunits in the generation of skeletal muscle Ca^{2+} channel activity (Lacerda *et al.*, 1991). Recently, the interaction of the β subunit with the α_1 subunit on the cytoplasmic side of the membrane has been suggested to play a role in the modulation of the Ca^{2+} current amplitude, inactivation process and the drug sensitivity of the skeletal muscle Ca^{2+} channel (Lory *et al.*, 1992).

The skeletal muscle γ subunit is very hydrophobic and highly glycosylated and is thought to be a transmembrane protein (Jay *et al.*, 1990). The effect of this subunit was modest on the Ca^{2+} current produced by the skeletal muscle α_1 subunit expressed in murine L cells (Varadi *et al.*, 1991). However, the co-expression of the skeletal muscle γ subunit increased the expression of Ca^{2+} current by the cardiac α_1 subunit in *Xenopus* oocytes and substantially modified the channel kinetics (Singer *et al.*, 1991; Catterall, 1991a).

1.4.5 Ligand Binding Sites on the Skeletal Muscle Ca^{2+} Channel Protein

1.4.5.1 High Affinity DHP-Binding Sites

VDCCs in the skeletal muscle (DHP binding proteins) carry binding sites for Ca^{2+} channel modulators belonging to different chemical classes. Initial studies involving covalent labelling of Ca^{2+} channels in skeletal muscle transverse tubules by DHPs indicated the binding sites to be present on the α_1 subunit (Ferry *et al.*, 1984; Galizzi *et al.*, 1986; Sharp *et al.*, 1987; Takahashi *et al.*, 1987; Vaghy *et al.*,1987; Catterall and Striessnig, 1992). Recent studies have been directed at identifying the region(s) on the α_1 subunit that bind to DHP and other classical Ca^{2+} channel ligands. Using the photoactive DHP analogues [^{3}H]azidopine and [^{3}H]nitrendipine Regulla *et al* (1991) showed that a region in the putative cytosolic domain adjacent to the sixth transmembrane helix of the fourth homologous domain (IV6) of the α_1 subunit carries the DHP binding site. This suggests that the DHP binding site is located on the intracellular side. This is also in agreement with the hydrophobicity of the DHPs and consistent with the "membrane bilayer pathway" model described by Rhodes *et al* (1985). According to this model, the DHPs partition into the membrane bilayer before rapidly diffusing to their binding sites. However, there is some contradictory electrophysiological evidence that suggest that the DHP binding sites are exposed on the extracellular side of the membrane (Kass and Arena, 1989; Kass *et al.*, 1991; Strubing *et al.*, 1993).

In experiments using the ionized, and presumably membrane impermeant, DHP derivatives (amlodipine and SDZ 207-180), Kass *et al* (1991) reported that these drugs can modulate Ca^{2+} channel activity only when applied extracellularly and were not effective from the intracellular side. Recently two studies, using photolabelling techniques and subsequent sequence-directed antibody mapping of the proteolytic fragments (Nakayama *et al.*, 1991; Striessnig *et al.*, 1991), have proposed the DHP binding site to be in a location predicted to be close to the extracellular side of the membrane. The DHP binding regions were mapped to that portion of the loop linking the fifth (S5) and the sixth helix (S6) of the third domain that forms the outer mouth of the channel, and also to the transmembrane segment 6 (S6) in the domains III and IV. Thus, the extracellular regions of the transmembrane segments in two separate but adjacent domains are believed to contribute to the formation of DHP binding site of the skeletal muscle Ca^{2+} channel (Catterall and Striessnig, 1992).

1.4.5.2 Phenylalkylamine and Benzothiazepine Binding Sites

Similar to the DHP binding sites, phenylalkylamine (e.g. verapamil) binding sites have also been identified on the α_1 subunit using photoreactive ligands such as ludopamil (Striessnig *et al.*, 1987; Sieber *et al.*, 1987). While the exact location of the DHP binding site has not yet been clearly established, the available evidence suggests the phenylalkylamine binding site to be accessible from the intracellular surface. In

reconstitution studies using planar bilayers, it was shown that D890, a charged derivative of verapamil, was effective only when applied to the intracellular side of the channel (Affolter and Coronado, 1986). Using antipeptide antibodies, the peptide fragment of the α_1 subunit labelled by the photoactive derivative of verapamil was found to lie between Glu-1349 and Trp-1391, a segment that includes the intracellular end of the sixth helix in the fourth homologous domain (IVS6) and the adjacent intracellular amino acid residues (Striessnig *et al.*, 1990). The inhibition of Ca^{2+} channels by phenylalkylamine ligands is accelerated by depolarization that opens the channels (use-dependent effect) (Lee and Tsien, 1983; Hondegham and Katzung, 1984). This suggests that the phenylalkylamine binding site is located within the intracellular opening of the channel pore, and a high affinity receptor-ligand interaction occurs only when the channel is open (Catterall and Striessnig, 1992). Since the binding sites for DHPs and phenylalkylamines are thus proposed to be located at the opposite ends of transmembrane helix IVS6, the allosteric interaction between these two sites possibly involves conformational changes induced by the movement of this transmembrane IVS6 segment (Nakayama *et al.*, 1991). Though little is known about the benzothiazepine (e.g. diltiazem) binding site on the VDCC, recent photoaffinity labelling studies have indicated that this site, like DHP and phenylalkylamine sites, is also located on the α_1 subunit (Naito *et al.*, 1989; Striessnig *et al.*, 1990a). Based on the effect of a membrane-impermeable, permanently charged diltiazem-like drug, SQ 32,428 on the Ca^{2+} channel activity in smooth muscle and skeletal muscle like cell lines, Hering *et al* (1993) have suggested the benzothiazepine

binding domain to be exposed to the extracellular surface of the L-type Ca^{2+} channel.

1.4.5.3 Low Affinity DHP-Binding Sites

In addition to the well described high affinity binding sites, there is also evidence for the presence of distinct low affinity DHP binding sites on, or related to, the high affinity DHP binding proteins in skeletal muscle transverse tubules. The presence of these sites was first revealed by the ability of micromolar concentrations of unlabelled DHPs to accelerate the rate of dissociation of DHP antagonist [^{3}H]PN200-110 from its high affinity site (Dunn and Bladen, 1991). More recently, these low affinity sites were further characterised using the fluorescent DHP ligand, felodipine, whose fluorescence changes upon its binding to transverse tubule membranes (Dunn and Bladen, 1992). The binding of this ligand to the transverse tubules results in a saturable enhancement of its fluorescence which can be excited either directly or indirectly via energy transfer from membrane proteins. This fluorescence enhancement can be inhibited, in a competitive manner, by micromolar concentrations of DHP agonists and antagonists.

The functional significance of these low affinity DHP binding sites has been described both in cardiac muscle and skeletal muscle (Brown *et al.*, 1986; Ohkusa *et al.*, 1991; Lamb, 1992). Brown *et al* (1986) examined the effects of DHP agonists and antagonists on Ca^{2+} channel currents in guinea-pig ventricular cells. Nitrendipine

exhibited a dual effect, stimulatory or inhibitory, depending on the holding potential. On the other hand, the stimulatory effect of Bay K8644 on Ca^{2+} current was mono- or biphasic depending on the test potential. These results were attributed to the presence of two distinct DHP binding sites associated with the Ca^{2+} channel - the stimulatory effect of DHPs being mediated by low affinity sites whereas the high affinity sites participate in either stimulatory or inhibitory effects depending on the membrane potential (Brown *et al.*, 1986). Recently, Ohkusa *et al* (1991) used isolated microsomal vesicles consisting of the transverse tubule-sarcoplasmic reticulum complex as an *in vitro* model to study the depolarization-induced Ca^{2+} release from the sarcoplasmic reticulum. DHP antagonists, nifedipine and nimodipine at micromolar concentrations reduced only the slow phase of Ca^{2+} release that is mediated by DHP-sensitive proteins (Ohkusa *et al.*, 1990). On the other hand, the fast phase- and caffeine-induced Ca^{2+} release were not affected. This functional effect of DHP antagonists paralleled their binding to low affinity, high capacity sites (K_d = 1.5 μM, B_{max} = 107 pmol/mg) (Ohkusa *et al.*, 1991).

1.5 FUNCTIONAL ROLE OF DHP-SENSITIVE Ca^{2+} CHANNELS IN SKELETAL MUSCLE

Cytoplasmic Ca^{2+} is an important regulator of contractile protein activation and tension generation in smooth, skeletal and cardiac muscle. In skeletal muscle, the surface membrane excitation results in the inward spread of the depolarizing signal

through the transverse tubule system which in turn leads to release of Ca^{2+} from the internal stores of the sarcoplasmic reticulum and subsequently the activation of contractile proteins (Sandow, 1952). The mechanism linking the depolarization of transverse-tubule membrane and the release of Ca^{2+} is still largely unknown. In the past decade, major insights have been gained on the molecular mechanism of e-c coupling with the identification, isolation, and sequencing of two key molecules involved in this signal transduction process: a DHP binding protein (DHPBP) which is located on and reacts to changes in the membrane potential across the transverse-tubule membrane, and a ryanodine receptor which contains the Ca^{2+} release channel in the sarcoplasmic reticulum (Dulhunty, 1992). The skeletal muscle transverse tubule membrane is the richest source of DHPBPs (Fosset *et al.*, 1983). Yet, paradoxically, the activation of e-c coupling in skeletal muscle appears to be independent of the influx of extracellular Ca^{2+} (Armstrong *et al.*, 1972; Lüttgau and Spiecker, 1979; Caputo, 1981; Cota and Stefani, 1981; Gonzalez-Serratos *et al.*, 1982; Frank, 1982; McCleskey, 1985; Lüttgau *et al.*, 1986). Further, the VDCCs present in the transverse tubule membranes activate very slowly, reaching peak current amplitude after hundreds of milliseconds, whereas the mechanical transients initiated by action potentials (that last only 2-3 msec) last only tens of milliseconds (Beaty *et al.*, 1987). In addition, some Ca^{2+} channel blockers, at concentrations that are sufficient to block the inward Ca^{2+} current do not inhibit the contractions (Gonzalez-Serratos *et al.*, 1982) and voltage-clamp pulses to above the equilibrium potential for Ca^{2+} can still induce Ca^{2+} release from the sarcoplasmic reticulum (Brum *et al.*, 1988). These

results suggest that inward Ca^{2+} currents are not essential for the activation of twitch contraction in skeletal muscle. On the other hand, Ca^{2+} influx through slow Ca^{2+} channels has been suggested to play a role in the development of contractile responses resulting from prolonged membrane depolarizations (Sanchez and Stefani, 1978; Potreau and Raymond, 1980; Frank, 1984; Jacquemond and Rougier, 1990; Oz and Frank, 1991; Frank and Oz, 1992), and that such responses are sensitive to blockade by Ca^{2+} channel blockers (Frank, 1984; Ildefonse *et al.*, 1985; Rakowski *et al.*, 1987; Ríos and Brum, 1987; Dulhunty and Gage, 1988; Oz and Frank, 1991).

1.5.1 Role of DHPBPs as Voltage-Sensor v/s VDCC in Skeletal Muscle

Skeletal muscle Ca^{2+} currents are small when compared with the density of proteins that bind the 1,4-DHP class of Ca^{2+} channel ligands (Schwartz *et al.*, 1985). This has prompted the speculation that only a small fraction of the DHP binding proteins function as Ca^{2+} channels (Schwartz *et al.*, 1985). According to this view, the majority of the DHP binding proteins would perform as voltage sensors of e-c coupling and only a small proportion would constitute functional Ca^{2+} channels. However, it has been pointed out that the small Ca^{2+} current seen is the consequence of the low open channel probability (0.08) of the Ca^{2+} channels in skeletal muscle (Coronado and Affolter, 1986; Lamb and Walsh, 1987; Lamb, 1991, 1992). Ma *et al* (1991) have confirmed this for transverse tubule membrane Ca^{2+} channels reconstituted into lipid bilayers. The probability of channel opening (P_o) was

very low even when the channels were maximally activated by voltage and in the presence of the agonist, Bay K8644 (Ma *et al.*, 1991).

Currently, it is viewed that the DHPBPs in skeletal muscle serve a dual role, acting as Ca^{2+} channels, and as voltage-sensors that, by mechanism(s) not yet clear, transmit the depolarizing signal across the transverse tubule membrane to the terminal cisterna of the sarcoplasmic reticulum triggering it to release Ca^{2+} (Ríos and Brum, 1987). The evidence supporting this dual role of DHPBPs was obtained from experiments on cultured skeletal muscle cells from mice with muscular dysgenesis that are unable to contract and substantially lack slow Ca^{2+} currents (discussed above) (Tanabe *et al.*, 1988; Adams *et al.*, 1990). Injection of expression vectors encoding the functional α_1 subunit of DHPBP into such deficient muscle cells results in recovery of slow Ca^{2+} currents and e-c coupling suggesting that the same protein can function as both Ca^{2+} channel and voltage-sensor (discussed above, section 1.4.3).

Alternatively, two different forms of the α_1 subunit have been suggested to perform the two roles. In skeletal muscle, >90% of the DHPBP α_1 subunit is truncated at the C-terminus to give a molecular mass of 165- 170 kDa, whereas only about 10% of the α_1 subunit is 212 kDa form as predicted from cloning studies. Based on these observations, DeJongh *et al* (1989) suggested that the larger form of the protein functions as Ca^{2+} channel whereas the abundantly present smaller form acts as voltage-sensor (Catterall, 1991). However, whether this is true has yet to be

established and there is considerable argument against this proposal. In studies where purified DHPBP were reconstituted as functional Ca^{2+} channels in bilayers, the protein appeared to contain only the low molecular weight form of the α_1 subunit (Gutierrez *et al.*, 1991). Further evidence supporting the idea that a single class of DHPBP can function as Ca^{2+} channel and as voltage-sensor was obtained in a recent study by Beam *et al* (1992). Expression of a complementary DNA encoding the protein that corresponds to the shorter (truncated) DHPBP (that was suggested to be abundantly present and function as voltage sensor; DeJongh *et al*, 1989) in dysgenic myotubes could restore both e-c coupling and the slow Ca^{2+} current.

In experiments where $^{45}Ca^{2+}$ flux was measured through purified DHPBPs reconstituted into lipid vesicles, only about 3% of the purified proteins were proposed to form functional Ca^{2+} channel (Curtis and Catterall, 1986). However, this may have been the result of purification and reconstitution procedures where only a fraction of the proteins retains its functional properties. Furthermore, the proportion of functional Ca^{2+} channels in reconstituted vesicle preparations can be increased by about 10 fold by phosphorylation (Nunoki *et al.*, 1986) suggesting that a substantial fraction of DHPBPs can function as Ca^{2+} channels (Lamb, 1992). Also, in cardiac muscle, where all DHPBPs are believed to form functional Ca^{2+} channels (Lew *et al.*, 1991), most of the α_1 subunits have smaller molecular mass of about 195 kDa than that predicted by cloning studies (Schneider and Hofmann, 1988; Mikami *et al.*, 1989).

The voltage-dependent Ca^{2+} channel activity of DHP binding proteins can be demonstrated using isolated transverse tubule membrane vesicles (Dunn, 1989; Bhat *et al.*, 1992). Transverse tubule membranes, when isolated, form sealed vesicles with inside-out orientation. While the transverse tubule membrane system of intact skeletal muscle is inaccessible to direct electrophysiological recording, isolated vesicle preparations provide direct access to the DHP binding protein population (Dunn, 1989). Transverse tubule membranes are capable of generating and maintaining membrane potential in response to potassium diffusion gradients established in the presence of K^+ ionophore, valinomycin. These vesicles can be loaded with $^{45}Ca^{2+}$ and its flux across the membrane can be quantitated by experimentally changing the membrane potential (Dunn, 1989; Bhat *et al.*, 1992). $^{45}Ca^{2+}$ flux stimulated by depolarization has been shown to be blocked by both inorganic and organic Ca^{2+} channel blockers and stimulated by DHP Ca^{2+} channel agonists. Thus, isolated transverse tubule membranes can be used to study the relationship between the properties of ligand binding and their effects on the functional responses of channels (Dunn, 1989).

Thus, each DHPBP may function both as voltage-sensor and as a Ca^{2+} channel. As voltage-sensor, these proteins may directly control voltage-dependent Ca^{2+} release which triggers twitch contractions that last only a few msec during which the channels need not open. As Ca^{2+} channels, the DHPBPs have slow activation kinetics and low open channel probability. This can be explained by the fact that

because of their high density in the transverse tubule membrane, there have to be inherent mechanisms present to reduce the Ca^{2+} influx through these channels to avoid toxic overloading of the cell with Ca^{2+} (Lamb, 1992). The Ca^{2+} entry through these channels may occur to counter the long-term depletion of internal Ca^{2+} stores and perhaps also to provide additional source of Ca^{2+} during prolonged contractions such as those that occur in tetanic responses (Oz and Frank, 1990; 1991; Lamb, 1992; Oba *et al.*, 1993). Furthermore, direct activation of contractile proteins by Ca^{2+} entering through the slow Ca^{2+} channels has been suggested (Jacquemond and Rougier, 1990; Blaineau *et al.*, 1993).

1.6 MODULATION OF SKELETAL MUSCLE VDCCs

The modulation of the activity of VDCCs by neurotransmitters and hormones may occur through either direct interaction with the GTP-binding proteins or by second messenger-linked phosphorylation/dephosphorylation events (Hosey and Lazdunski, 1988; Miller and Fox, 1990). So far, the best understood mechanism of VDCC modulation is the effect of phosphorylation by cyclic AMP-dependent protein kinase induced by activation of β-adrenergic receptors (Brum *et al.*, 1983; Tsien *et al.*, 1986). Stimulation of cardiac Ca^{2+} channel activity by this mechanism involves phosphorylation of the channel that leads to an increase in the probability of channel opening (Trautwein and Pelzer, 1988). L-type Ca^{2+} channel activity in skeletal muscle is also positively modulated by mechanisms involving cAMP-dependent

phosphorylation (Schmid *et al.*, 1985; Arreola *et al.*, 1987). *In vitro* phosphorylation studies have demonstrated that both the α_1 and β subunits of skeletal muscle Ca^{2+} channels are phosphorylated by cAMP-dependent protein kinase (Curtis and Catterall, 1985; Hosey *et al.*, 1987). Recently, purified skeletal muscle Ca^{2+} channels incorporated into liposomes have been shown to be activated by either cAMP-dependent protein kinase (PKA) (Nunoki *et al.*, 1989; Chang *et al.*, 1991) or by exposure of the muscle to agents that activate cAMP system prior to channel isolation (Mundiña-Weilenmann *et al.*, 1991). In purified channels (Flockerzi *et al.*, 1986) or native channels from transverse tubule vesicles (Yatani *et al.*, 1988; Mundiña-Weilenmann *et al.*, 1991) that are reconstituted into planar bilayers, PKA greatly increases the probability of channel opening in addition to decreasing the rate of channel inactivation and increasing the availability of the channel.

Protein kinase C (PKC) has been suggested to regulate the appearance of high affinity DHP binding proteins in the skeletal muscle cells (Navarro, 1987). The α_1 subunit has been shown to be the preferred substrate for PKC and phosphorylation results in the activation of the channels (O'Callahan *et al.*, 1988; Chang *et al.*, 1991; Ma *et al.*, 1992) while the β subunit is phosphorylated to a lesser extent. In contrast to the effect of PKA (Mundiña-Weilenmann *et al.*, 1991), PKC increases the maximum open channel probability largely through a voltage-independent mechanism (Ma *et al.*, 1992).

Although skeletal muscle DHP binding proteins have been shown to be phosphorylated by other kinases such as Ca^{2+}/calmodulin dependent protein kinase (Hosey *et al.*, 1986) and an endogenous transverse tubule kinase (Imagawa *et al.*, 1987), their functional significance has yet to be established.

In addition to the second-messenger mediated effects, Ca^{2+} channels have also been shown to be regulated directly by guanine nucleotide binding (G) proteins (Yatani *et al.*, 1987; 1988; Brown *et al.*, 1989). In studies of skeletal muscle Ca^{2+} channels incorporated into bilayers, both an activated G protein (G^{*}_{s}) and a purified G protein α subunit (α_{s}^{*}) stimulated the channel open probability by about 13-25 fold (Yatani *et al.*, 1988). Recently, β-adrenergic receptor mediated inhibition of L-type Ca^{2+} current in cultured skeletal muscle cells has been shown to involve a pertussis toxin-sensitive G protein (Somasundaram and Tregear, 1993). There is also pharmacological and biochemical evidence to support the direct association of the skeletal muscle Ca^{2+} channel with G proteins. The nonhydrolysable analog of GTP (GTPγS) has been shown to affect binding of Bay K8644 to its low affinity sites (Dunn and Bladen, 1991). Furthermore, Ca^{2+} channels and G proteins have been shown to be closely associated in the transverse tubule membrane system (Toutant *et al.*, 1990; Hamilton *et al.*, 1991) supporting the idea that the skeletal muscle Ca^{2+} channels may be direct effectors for G proteins.

2. STATEMENT OF THE PROBLEM

Skeletal muscle transverse tubule (t-tubule) membranes contain the highest density of proteins that bind the 1,4-dihydropyridine (DHP) class of Ca^{2+} channel ligands (Fosset *et al.*, 1983; Glossmann *et al.*, 1983). Although these proteins function as voltage-dependent Ca^{2+} channels, the functional significance for their abundance in skeletal muscle remains unclear (Lamb, 1992). While cardiac- and smooth muscle contraction require the influx of extracellular Ca^{2+}, the activation of contraction in skeletal muscle is independent of extracellular Ca^{2+} (Armstrong *et al.*, 1972). The VDCCs that are present in the skeletal muscle t-tubule membrane system activate slowly, reaching peak conductance only after about 200 msec, whereas an action potential triggering the twitch type of contraction lasts only about 2 msec (Chiarandini and Stefani, 1983; Beaty *et al.*, 1987). Currently, it is believed that DHP binding proteins in skeletal muscle play a dual role, acting both as "voltage-sensors" and as VDCCs (Ríos and Brum, 1987; Beam *et al.*, 1987). As voltage-sensors, these proteins may rapidly activate twitch contractions by their ability to communicate the membrane depolarization to the sarcoplasmic reticulum to trigger the rapid release of Ca^{2+}. As VDCCs, the DHP binding proteins may play an active role in more prolonged contractile responses such as tetanic responses by providing an additional source of Ca^{2+} to the contractile proteins (Oz and Frank, 1991; Blaineau *et al.*, 1993; Oba *et al.*, 1993).

An understanding of the molecular properties of skeletal muscle DHP binding proteins relies on their characterization in isolation or in a defined system. This includes examination of their ability to transport Ca^{2+} and to function as voltage-sensing molecules. The inaccessibility of the t-tubule system to direct electrophysiological manipulation *in vivo* has limited our understanding of the functional roles of these proteins, although they may be studied after purification and reconstitution into vesicles or planar lipid bilayer (Affolter and Coronado, 1985; Flockerzi *et al.*, 1986; Ma *et al.*, 1991). Isolated t-tubule membranes provide an ideal *in vitro* model to study the VDCC activity of DHP-binding proteins in their native membrane environment. When isolated, these membranes form sealed vesicles with an inside-out orientation (Hidalgo *et al.*, 1986), and their membrane potential can be experimentally manipulated (Dunn, 1989).

A $^{45}Ca^{2+}$ flux assay technique has been developed to measure the VDCC activity in these isolated t-tubule membranes on slow time scales of 20 sec (Dunn, 1989). However, since the VDCCs in skeletal muscle operate on a subsecond time scales, it is important to study channel function on time scales of physiological relevance. In the present study, therefore, an automated rapid filtration technique has been developed to measure voltage-dependent $^{45}Ca^{2+}$ efflux responses on a millisecond time scale. The kinetic properties of the VDCCs, and their modulation by Ca^{2+} channel ligands has been examined.

Even though 1,4-DHP ligands bind to DHP binding proteins in the skeletal muscle with high affinity (apparent K_d of nanomolar concentrations), these drugs are pharmacologically effective only in the micromolar range. This discrepancy may be explained by the interaction of DHP ligands with distinct low affinity binding sites in skeletal muscle (Dunn and Bladen, 1992). An attempt has been made, in the present study, to examine the possible involvement of these low affinity binding sites in regulating voltage-dependent Ca^{2+} channels in skeletal muscle.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Solutions

All solutions were prepared fresh in distilled and deionized water, and kept at 4°C where appropriate. The compositions of the buffer solutions used in this study are given in the Methods section. All buffers contained the antibacterial preservative sodium azide (NaN_3) at 0.02% (w/v). Where indicated, the buffers contained the following protease inhibitors at stated concentrations: iodoacetamide, 1 mM; phenylmethylsulphonyl fluoride (PMSF), 0.1 mM; benzamidine, 0.1 mM; leupeptin, 1 μg/ml; pepstatin A, 1 μM.

3.1.2 Drugs and Chemicals

Drugs used in this study were:

(a)	Nifedipine	Sigma Chemical Company
(b)	Nitrendipine	Miles Laboratories Inc. New Haven, CT.
(c)	(±)Bay K8644	Miles Laboratories Inc. New Haven, CT.
(d)	Amlodipine	Pfizer Limited.
(e)	Trypsin	Sigma Chemical Company
(f)	[^{3}H]PN200-110	Dupont, New England Nuclear
(g)	$^{45}Ca^{2+}$	Dupont, New England Nuclear or ICN
(h)	Valinomycin	Sigma Chemical Company
(i)	A23187	Sigma Chemical Company

All drugs were freshly prepared in the appropriate buffer except nifedipine,

nitrendipine and (±)Bay K8644 which are prepared in dimethyl sulphoxide (DMSO). In experiments where these drugs were used, all samples, including controls, contained DMSO at the same final concentration (not exceeding 0.2%) (Dunn, 1989).

3.2 METHODS

3.2.1 Preparation of Microsomal Membranes

Microsomal membranes were prepared from the back and hind leg muscles from young adult New Zealand White rabbits (weighing 2-3 lbs.) using previously described methods (Dunn, 1989). All procedures were carried out at 0-4°C to prevent proteolytic degradation of proteins. The excised muscles were cut into small pieces and added into four volumes of homogenization buffer (20 mM Tris-HCl; 0.3 mM Sucrose; 5 mM EDTA; 1.2 mM EGTA; pH, 7.4) containing protease inhibitors. The mixture was homogenized using five 20-sec high speed bursts in a Waring commercial blender. Following centrifugation of this homogenized muscle preparation for 20 min at 4000 x g_{max} (5000 rpm in a Sorvall GSA rotor), the pellet was discarded and the supernatant was recentrifuged for 20 min at 10,500 x g_{max} (8000 rpm in a Sorvall GSA rotor). The supernatant was filtered through six layers of cheese cloth to separate fat particles, and the contractile proteins were solubilized by adding solid potassium chloride to a final concentration of 0.5 M. This mixture was stirred for 30 min at 4°C and then centrifuged for 45 min at 186,000 x g_{max} (40,000 rpm in a Beckman Type 45 Ti rotor). The resulting microsomal membrane pellets were resuspended in homogenization buffer using two or three 20 sec bursts of a Virtis-45

homogenizer at a setting of 50. The membranes were washed twice by centrifugation at 186,000 x g_{max} and resuspension in homogenization buffer. The microsomal membranes were finally resuspended in resuspension buffer (20 mM Tris-HCl; 15% sucrose w/v; pH, 7.4) and used immediately to purify transverse tubule membranes or frozen in liquid nitrogen and stored at -80°C for future use.

3.2.2 *Purification of Transverse Tubule (T-tubule) Membranes*

Transverse tubule (t-tubule) membranes were prepared from microsomal membranes using discontinuous sucrose density gradient differential centrifugation (Dunn, 1989). Sucrose gradients were prepared with three layers of 10 ml volumes each of 35% (w/v), 27.5% (w/v) and 25% (w/v) sucrose in Tris buffer (20 mM Tris-HCl; pH, 7.4). Microsomal membranes (6ml/gradient) in resuspension buffer (20 mM Tris-HCl; 15% sucrose w/v; pH, 7.4) were layered on top of the gradients. Following centrifugation at 20,000 rpm for 18 hours in a Beckman SW28 rotor at 4°C, the floating top layer was discarded and the cloudy band at the 15-25% sucrose interface containing transverse tubules was collected by aspiration. The sucrose in this fraction was diluted by adding Tris buffer (20 mM Tris-HCl; pH, 7.4) drop-wise to the sample while continuously stirring on ice. The t-tubule membranes thus diluted were collected by centrifugation at 186,000 x g_{max} for 45 min at 4°C. The resulting pellet, containing t-tubule membranes, was resuspended in appropriate buffer using two 20-sec bursts of the Virtis-45 homogenizer at setting 50.

In order to equilibrate the t-tubule membranes in low potassium equilibration buffer (10 mM Hepes-Tris; pH, 7.4; 145 mM choline chloride; 5 mM potassium gluconate), the membranes were diluted in a large volume of this buffer and washed at least twice by centrifugation and resuspension. Following final resuspension in a small volume (0.5-1.0 ml) of the same buffer, the membranes were frozen in liquid nitrogen and slowly thawed at 4°C and this freeze-thaw cycle was repeated at least once. This freeze-thaw cycle has been shown to be sufficient for the equilibration of intra- and extravesicular ions (Dunn, 1989). The membranes were frozen in liquid nitrogen and stored at -86°C until future use, or stored on ice prior to immediate use. Protein concentration in the membrane preparations was measured by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA.).

The biochemical properties of purified t-tubule membranes have been extensively studied (Rosemblatt et al., 1981; Hidalgo et al., 1986; Dunn, 1989). A large fraction (>90%) of the t-tubule membranes prepared from frog and rabbit skeletal muscle remain as sealed vesicles in an inside-out orientation (Hidalgo et al., 1986; Dunn, 1989).

3.2.3 Receptor-Binding Assays

3.2.3.1 Equilibrium Binding of [^{3}H]PN200-110 to T-tubule Membranes

In all the t-tubule membrane preparations, the binding activity of [^{3}H]PN200-110 was measured. The equilibrium binding experiments were carried out by

filtration assay under subdued lighting conditions to minimize ligand photolysis. Aliquots of t-tubule membranes (200 μl) were added to different concentrations [0.2-2.0 nM] of radiolabelled ligand to give a final protein concentration of 0.04 mg/ml in a total volume of 0.4 ml receptor binding assay buffer (25 mM Hepes-Tris; pH, 7.4; 1 mM $CaCl_2$). Following a 60 min incubation in the dark at room temperature, 0.2 ml aliquots of each sample were removed and rapidly filtered under vacuum through Whatman GF/C filters using a Hoefer manifold filtration apparatus. The filters were immediately washed with two 5 ml aliquots of ice cold assay buffer. After drying, the filters were extracted in 5 ml of ACS (Amersham, Canada) scintillation fluid and counted for ^3H radioactivity. Duplicate 50 μl aliquots of the incubation mixtures were counted directly for estimating the total ligand concentration. Non-specific binding of radiolabelled ligand was estimated from parallel measurement of binding in the presence of 1 μM of unlabelled ligand, nitrendipine.

3.2.3.2 *Membrane Potential Dependence of DHP Binding to T-tubule Membranes*

The membrane potential dependence of DHP binding was examined by measuring the kinetics of association of [^3H]PN200-110 binding using low concentration of membranes and excess ligand. This protocol was used so that equilibrium between the receptor and the ligand is reached faster during which time the membrane potential that has been established does not dissipate. T-tubule membranes in low potassium equilibration buffer (see above) were diluted to 0.2

mg/ml and loaded with 5 mM unlabelled $CaCl_2$ by two freeze thaw cycles to mimic the conditions used for $^{45}Ca^{2+}$ flux experiments (described below). These membranes were further diluted to 0.01 mg/ml in buffers (10 mM Hepes-Tris; pH, 7.4) containing appropriate concentrations of potassium gluconate with isosmotic exchange of choline chloride, 0.166 μM EGTA and 0.1 μM valinomycin to generate the desired extravesicular potential, and incubated at room temperature for 5 min. To initiate association, [^{3}H]PN200-110 was added to a final concentration of 5 nM and at appropriate intervals (from 0 to 25 min), 0.5 ml aliquots were filtered through Whatman GF/C filters using a Hoefer manifold filtration apparatus. After drying, the filters were extracted in 5 ml ACS (Amersham, Canada) scintillation fluid and counted for ^{3}H radioactivity. Duplicate 100 μl aliquots from the incubation mixture were counted directly to estimate the total ligand concentration.

3.2.3.3 Dissociation of [^{3}H]PN200-110 from its Binding Sites: Effect of Amlodipine

T-tubule membranes (0.05 mg/ml) in assay buffer (25 mM Hepes-Tris; pH, 7.4; 1 mM $CaCl_2$) were incubated with 1 nM [^{3}H]PN200-110 at room temperature for 45 min. Dissociation was initiated by 20 fold dilution of the membranes into the same buffer alone or buffers containing different concentrations of amlodipine. At intervals (from 0-60 min) 1 ml aliquots were filtered through Whatman GF/C filters using a Hoefer manifold filtration apparatus and the filters were washed with two 5 ml volumes of ice cold assay buffer (with or without amlodipine). The filters were dried

and extracted with 5 ml ACS (Amersham, Canada) scintillation fluid before counting for [^{3}H]. Non-specific binding was estimated by incubating membranes with excess unlabelled nitrendipine.

3.2.3.4 Fluorescence Experiments with Amlodipine

All fluorescence experiments were carried out using a Perkin-Elmer MPF4 fluorometer thermostated at 25°C. Tris buffer (20 mM Tris-HCl; pH, 7.4) containing 1 mM $CaCl_2$ was used in all the experiments. The fluorescence emission spectrum of amlodipine was recorded using an excitation wavelength of either 370 nm (direct excitation) or 290 nm (energy transfer from t-tubule membrane proteins). Quartz cuvettes were used in all experiments.

In titration experiments, aliquots of amlodipine from stock solutions prepared in 20 mM Tris-HCl, pH, 7.4; 1 mM $CaCl_2$ were added to 2 ml of buffer alone or buffer containing t-tubule membranes (0.01 mg/ml) and the samples were continuously stirred. Fluorescence readings were recorded immediately after each addition, and the excitation shutter was closed between readings to minimize photolysis of amlodipine. The specific enhancement of fluorescence at each concentration of amlodipine was calculated by subtracting the fluorescence reading of amlodipine in buffer from the fluorescence observed in the presence of t-tubule membranes.

In competition experiments, 2 ml of t-tubule membranes (5 μg/ml) were incubated with 5 μM amlodipine for 15 min in a quartz cuvette at 25°C. Aliquots of concentrated solution of (±)Bay K8644 prepared in DMSO were added and the amlodipine fluorescence reading was recorded using direct excitation at 370 nm. The spectral interference occurring due to the presence of added (±)Bay K8644 was corrected by parallel titration of amlodipine with (±)Bay K8644 in the absence t-tubule membranes.

3.2.4 $^{45}Ca^{2+}$ Efflux Assays

Because of the inside-out orientation of the sealed t-tubule membrane vesicles, depolarization-activated Ca^{2+} flux through the membrane-bound VDCCs was measured by monitoring efflux of $^{45}Ca^{2+}$ from preloaded vesicles. All the efflux assays were carried out at room temperature.

3.2.4.1 Loading of Membrane Vesicles with $^{45}Ca^{2+}$

The t-tubule membrane vesicles in low potassium equilibration buffer (see above) were loaded with $^{45}Ca^{2+}$ by the addition of one-half volume of isotopically diluted $^{45}CaCl_2$ solution in the same buffer to give a final total $CaCl_2$ concentration of 5 mM containing approximately 50 μCi/ml $^{45}Ca^{2+}$. The mixture was subjected to two cycles of rapid freezing in liquid nitrogen and slow thawing at 4°C (Moore and Raftery, 1980; Dunn, 1989). The final membrane protein concentration was maintained at about 0.2 mg/ml. The $^{45}Ca^{2+}$ loaded vesicles were kept on ice and

used within 2-3 hours.

3.2.4.2 *Repolarization of the T-tubule Membranes*

The $^{45}Ca^{2+}$-loaded membrane vesicles in low potassium buffer were diluted 40 fold in high potassium repolarizing buffer (10 mM Hepes-Tris; pH, 7.4; 150 mM potassium gluconate; 0.166 μM EGTA; 0.1 μM valinomycin). This dilution in high K^+ buffer mimics the resting state of the cell by generating an estimated outside membrane potential of -86 mV. The extravesicular concentration of Ca^{2+} was reduced to less than 0.1 μM to mimic the intracellular Ca^{2+} concentration by including EGTA in this buffer. Valinomycin (0.1 μM) included in the high potassium buffer facilitates the development of K^+ gradient across the vesicular membrane. The repolarization process was allowed to proceed for 3 min before initiating the efflux response.

3.2.4.3 *Measurement of $^{45}Ca^{2+}$ Efflux*

Initiation and measurement of $^{45}Ca^{2+}$ efflux from preloaded vesicles that have been repolarized involves removing high potassium buffer from the extravesicular space and washing with depolarizing low potassium buffer (10 mM Hepes-Tris; pH, 7.4; 145 mM choline chloride; 5 mM potassium gluconate; 0.166 μM EGTA; 0.1 μM valinomycin) for a predetermined time period and measuring the quantity of $^{45}Ca^{2+}$ that is retained inside the vesicles.

In experiments where the manual filtration technique was used, after 3 min of repolarization, an aliquot (0.9 ml) of $^{45}Ca^{2+}$-loaded t-tubule membrane vesicles was applied to a Whatman GF/C filter which had been pre-equilibrated in the same buffer and mounted in a Hoefer manifold filtration apparatus, and the extravesicular buffer was removed under vacuum. 1 ml of depolarizing low potassium buffer was then added. This depolarizes the membranes to an estimated 0 mV to stimulate efflux of $^{45}Ca^{2+}$ from inside the vesicles. The efflux was allowed to continue for 20 sec before the depolarizing buffer was removed under vacuum and the filters were rapidly washed with two 5 ml volumes of "stop" buffer (10 mM Hepes-Tris; pH, 7.4; 5 mM potassium gluconate; 145 mM choline chloride; 0.1 mM lanthanum chloride; 30 mM sucrose). The filters with the adsorbed membrane vesicles were dried, extracted with 5 ml of ACS (Amersham, Canada) scintillation fluid, and counted for the $^{45}Ca^{2+}$ retained in the vesicles.

In experiments where the $^{45}Ca^{2+}$ flux response was measured by depolarizing the membranes on millisecond time scales, a rapid filtration device (Biologic, Meylan, France) was used. This apparatus consists of a holder for a 25 mm diameter filter, a motor-driven injection device and a microprocessor (Dupont, 1984). An aliquot (0.9 ml) of the $^{45}Ca^{2+}$-loaded membrane vesicles were pipetted onto a Whatman GF/C filter (that was presoaked in the appropriate buffer and mounted in the filter holder), and the extravesicular buffer was removed under vacuum. Flux was initiated by starting the force-filtration of the filter with the depolarizing low

potassium buffer and the flux was allowed to proceed for predetermined time (30 msec to 10 sec). The filters were then dried and counted for residual $^{45}Ca^{2+}$ after extracting in 5 ml ACS (Amersham, Canada) scintillation fluid.

In experiments where the membranes were repolarized to different potentials, potassium gluconate was replaced with isosmotic choline chloride. In experiments where the effect of extravesicular Ca^{2+} was tested on the flux response, concentrations of EGTA were changed to chelate the extravesicular Ca^{2+} to the desired concentrations (Schilling and Lindenmayer, 1984). The free Ca^{2+} concentration was calculated by assuming that the Ca^{2+}/EGTA dissociation constant = 0.033 μM at pH 7.4 (calculated using a computer software, *Calcium*).

3.2.4.4 Drugs

In all $^{45}Ca^{2+}$ efflux assays, when indicated, the $^{45}Ca^{2+}$-loaded t-tubule membrane vesicles were pre-incubated with the appropriate drug or solvent vehicle for 30 min on ice before initiating the repolarization. All buffers used for repolarization and subsequent depolarization contained the drug (or solvent vehicle) at the same concentration.

3.2.4.5 Membrane Potentials

The values of the membrane potentials indicated in the text, figures and figure legends were predicted from the Nernst equilibrium potential for K^+ (at 25°C) using

the concentrations of potassium contained in the intra- and extravesicular buffers.

$$E_m \approx E_K \approx 58 \log [K_i/K_o]$$

where K_o = extravesicular K^+ concentration

and K_i = intravesicular K^+ concentration

All the values of membrane potentials given refer to those in cells with normal orientation, e.g. an outside negative membrane potential in the inside-out transverse tubule vesicles refers to the inside negative membrane potential in a cell with right side-out orientation.

3.2.5 Data Analysis

The arithmetic means and standard errors of the means (s.e.m.) of experimental results are presented. the number of experiments (n) carried out are indicated in the figure legends, where appropriate. The best-fit values for receptor-binding and fluorescence experiments, where indicated, were calculated using the computer software, GraphPad Inplot, version 4.0 (GraphPad Software, San Diego, CA).

4. RESULTS

4.1 Introduction

Skeletal muscle transverse tubules are the richest source of proteins that bind the 1,4-dihydropyridine class of L-type Ca^{2+} channel ligands (Fosset *et al.*, 1983; Glossmann *et al.*, 1983). This is consistent with the localization of voltage-dependent Ca^{2+} channels (VDCCs) in the transverse tubule system (Almers *et al.*, 1981; Chiarandini and Stefani, 1983). Despite their high density in the transverse tubules, the functional significance of these proteins in skeletal muscle is not yet clearly established. The transverse tubule system in intact skeletal muscle is not accessible to electrophysiological recordings because of the anatomic location of the transverse tubule system deep within the muscle fibre.

Isolated transverse tubule membranes have been used to measure the biochemical properties and functional characteristics of VDCCs in skeletal muscle (Dunn, 1989). When isolated, these membranes form sealed, inside-out vesicles. It has been demonstrated that the isolated transverse tubule membranes are capable of generating a membrane potential in response to establishing a potassium concentration gradient across the membrane (Dunn, 1989). VDCCs in isolated transverse tubule membranes remain largely inactivated, probably as a result of the prolonged depolarization that occurs during membrane isolation. This inactivation can be removed by repolarizing the membrane using experimental conditions that mimic the resting state of the cell. This repolarization is achieved by diluting the

membrane vesicles equilibrated in low potassium buffer into buffers containing high potassium. Subsequent washing of these vesicles with low potassium buffer results in membrane depolarization triggering the efflux of Ca^{2+} from preloaded vesicles. The development of the potassium gradient is facilitated by including the potassium ionophore, valinomycin in the buffers. In all $^{45}Ca^{2+}$ flux experiments, changes in potential across the transverse tubular membranes were induced by isosmotic exchange of choline chloride for potassium gluconate in buffers. Such replacement of both cations and anions has been shown to be effective in generating membrane potential and this prevents membrane vesicles from undergoing osmotic damage (Ikemoto *et al.*, 1984). Under these conditions where both choline (cation) and gluconate (anion) are impermeable through the membrane vesicles, the membrane potential across the transverse tubule membrane can be predicted by Nernst equilibrium potential for potassium (at 25°C), assuming that in the presence of potassium ionophore, valinomycin, the relative permeability of potassium is far greater than that of chloride.

$$E_m \approx E_K \approx 58 \log [K_i/K_o]$$

where K_o = extravesicular K^+ concentration

and K_i = intravesicular K^+ concentration

Using the potential sensitive dye, diS-C_3-(5), the transverse tubule vesicles have been shown to develop membrane potentials predicted by the Nernst equation (Dunn, 1989).

In the present study, isolated transverse tubule membrane vesicles have been used to further characterize the VDCC activity of DHP binding proteins in their native membrane environment. Voltage-dependent $^{45}Ca^{2+}$ flux responses have been measured on more physiologically relevant time-scales using an automated rapid filtration technique (Dupont, 1984).

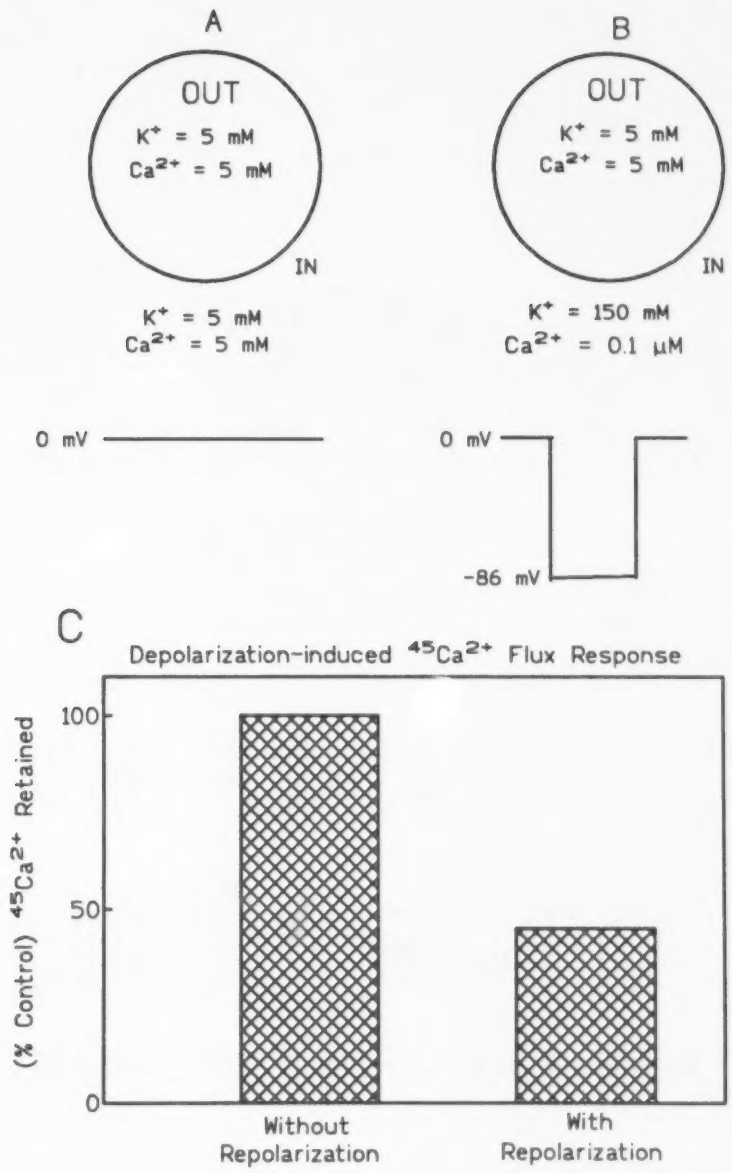
4.2 Measurement of $^{45}Ca^{2+}$ Flux Using Manual Filtration Technique

It was previously reported (Dunn, 1989) that isolated transverse tubule vesicles in low potassium (5 mM) buffer develop an estimated outward membrane potential of -86 mV when they are diluted into a buffer containing 150 mM K^+ in the presence of valinomycin. This condition, in an inside-out vesicles, mimics the resting state of a cell. Subsequent depolarization of the membranes by washing with low K^+ buffer induces efflux of $^{45}Ca^{2+}$ from preloaded vesicles and the efflux response may be stopped by rapid washing of the vesicles with a "stop" buffer containing 0.1 mM $LaCl_3$. Similar results were obtained in the present study. Transverse tubule vesicles were loaded with 5 mM total Ca^{2+} containing approximately 50 μCi/ml of $^{45}Ca^{2+}$ using two cycles of freezing in liquid nitrogen and slow thawing at 4°C. Most of the $^{45}Ca^{2+}$ that is trapped in the vesicles represent a releasable pool of $^{45}Ca^{2+}$ since almost complete efflux was observed when the vesicles were diluted into buffer containing 2 μM of the Ca^{2+} ionophore, A23187 (Figure 3). In studies of VDCC activity, the vesicles were repolarized for 3 min to an estimated -86 mV by 40 fold

dilution into buffer containing 150 mM potassium gluconate and 0.1 μM valinomycin. The extravesicular Ca^{2+} was chelated to approximately 0.1 μM by adding EGTA in the dilution buffer. At the end of 3 min repolarization, an aliquot of membranes was depolarized to approximately 0 mV for 20 sec by exposing the vesicles to buffer containing 5 mM potassium gluconate, and the reaction was stopped by rapid washing of vesicles with "stop" buffer. This two-step protocol, involving first repolarization and subsequent depolarization results in significant efflux of $^{45}Ca^{2+}$ from the vesicles. About 30% of the trapped $^{45}Ca^{2+}$ is effluxed under such conditions. This $^{45}Ca^{2+}$ flux presumably occurs through VDCCs since the flux response was completely inhibited by the presence of the inorganic VDCC blocker, La^{3+}. A schematic diagram of the "two-step" experimental protocol used to measure the $^{45}Ca^{2+}$ flux response is shown in figure 2. The results of the $^{45}Ca^{2+}$ flux experiments conducted using 20 sec depolarization are shown in figure 3.

It has been shown that the presence of valinomycin, a potassium ionophore,is important to facilitate the development of potassium diffusion potential across the vesicular membrane in order to see voltage-dependent $^{45}Ca^{2+}$ flux responses. When valinomycin was omitted from repolarizing buffer, there was no $^{45}Ca^{2+}$ efflux measured by subsequent depolarization. Thus, it is clear that the observed $^{45}Ca^{2+}$ flux response is the result of changes in the membrane potential caused by the potassium ion gradient.

Figure 2. Schematic diagram of the experimental protocol used to measure depolarization-induced $^{45}Ca^{2+}$ flux in transverse tubule membrane vesicles. The transverse tubule vesicles in low K^+ buffer loaded with $^{45}Ca^{2+}$ were diluted into either the same (non-repolarizing) buffer (A) or into repolarizing high K^+ buffer (B). After 3 min incubation, the vesicles were depolarized to an estimated 0 mV with low K^+ buffer for a predetermined time, and the amount of $^{45}Ca^{2+}$ retained by the vesicles was measured (C). Because of the inside-out orientation of the vesicles, the intravesicular space of the vesicles in the figure is referred to as OUT and the extravesicular space as IN.


A
OUT
K^+ = 5 mM
Ca^{2+} = 5 mM
IN
K^+ = 5 mM
Ca^{2+} = 5 mM
B
OUT
K^+ = 5 mM
Ca^{2+} = 5 mM
IN
K^+ = 150 mM
Ca^{2+} = 0.1 μM
0 mV
0 mV
-86 mV
C
Depolarization-induced $^{45}Ca^{2+}$ Flux Response
(% Control) $^{45}Ca^{2+}$ Retained
100
50
0
Without Repolarization
With Repolarization

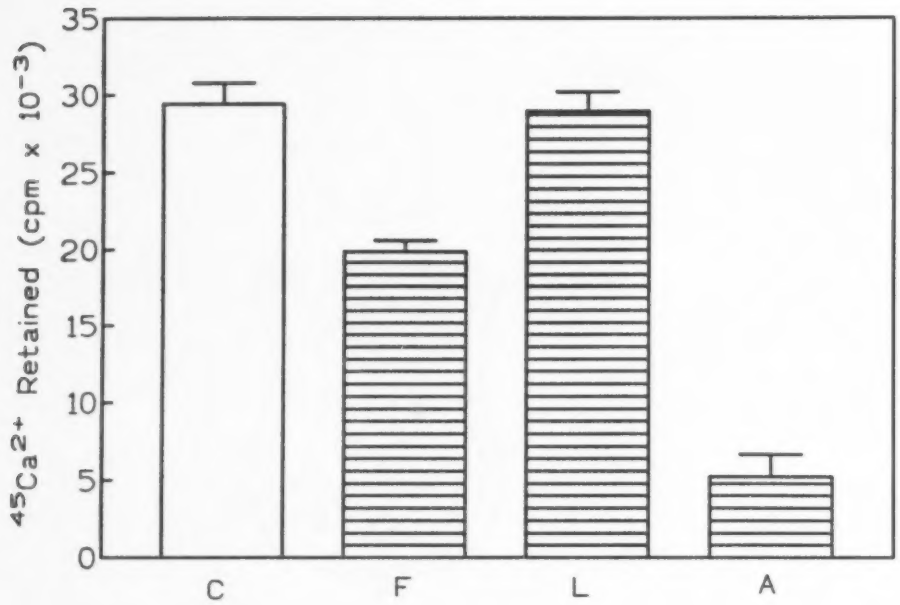


Figure 3. Depolarization-induced $^{45}Ca^{2+}$ efflux from transverse tubule vesicles. The vesicles equilibrated in low (5 mM) K^+ buffer and loaded with $^{45}Ca^{2+}$ were diluted into either the same buffer (0 mV, empty bar) or repolarized to an estimated -86 mV by diluting in 150 mM K^+ (Filled bars) containing no additions (F), 1 mM $LaCl_3$ (L), or 2 μM A23187 (A). After 3 min incubation at room temperature, an aliquot of membranes was depolarized to approximately 0 mV for 20 sec using the manual filtration technique as described in the Methods section. Bars indicate average ± s.e.m. (n=4).

4.3 Time Course of $^{45}Ca^{2+}$ Flux Response Measured by Rapid Filtration Technique

Depolarization-induced $^{45}Ca^{2+}$ flux response measured using manual filtration techniques may be difficult to interpret because the time scales (20 sec) used are much longer than the time scales of physiological responses. In skeletal muscle, VDCCs have slower activation kinetics than other tissues, and peak conductance is reached about 100-200 msec after the initiation of depolarization. Hence, it is important to measure the $^{45}Ca^{2+}$ flux response on similar time scales. A rapid filtration technique has, therefore, been developed to measure $^{45}Ca^{2+}$ flux on such rapid time scales. This technique enables depolarization-dependent responses to be measured in the range of 10 msec to 10 sec. An aliquot of repolarized membranes was applied to a GF/C filter mounted in the filter holder of the Biologic filtration device and the extravesicular buffer was removed under vacuum. The membranes were then depolarized for a predetermined time (from 30 msec to 10 sec) by force filtration with low K^+ (depolarizing) buffer. The results of such an experiment are presented in figure 4. The amount of $^{45}Ca^{2+}$ retained by the vesicles that are either repolarized or not repolarized before subsequent depolarization was measured and the difference between the two gives specific voltage-dependent $^{45}Ca^{2+}$ efflux.

Even in control samples that were not repolarized before depolarization, there is a considerable decrease in the amount of $^{45}Ca^{2+}$ retained by the vesicles with

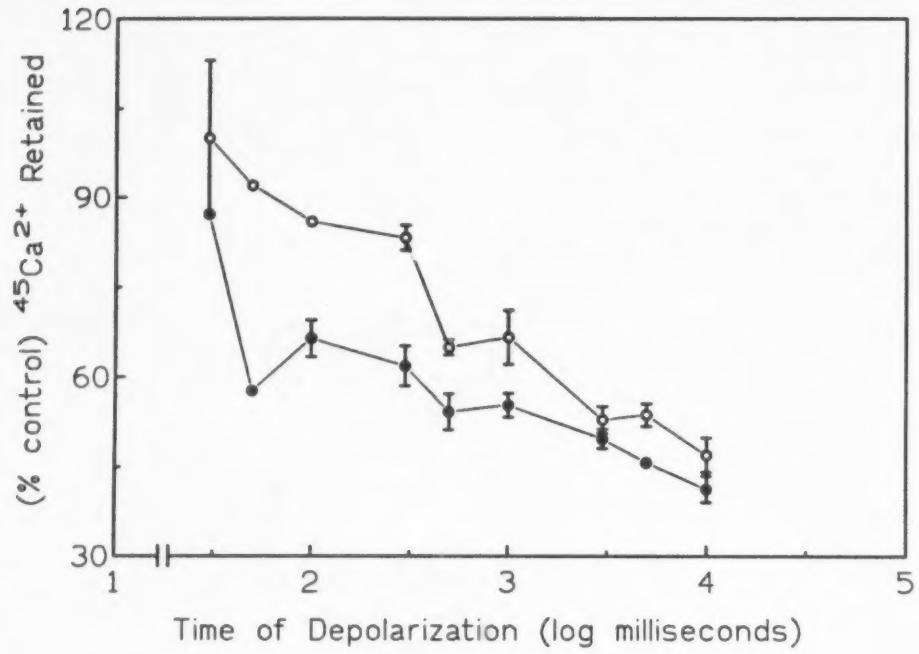


Figure 4. Time course of $^{45}Ca^{2+}$ flux response in transverse-tubule membrane vesicles. The vesicles equilibrated in low K^+ buffer and loaded with $^{45}Ca^{2+}$ were diluted into either the same buffer (○) or into a repolarizing buffer containing 150 mM K^+ (●) designed to generate an estimated outward membrane potential of -86 mV. After 3 min incubation at room temperature, an aliquot of vesicles was force filtered with buffer containing 5 mM K^+ to depolarize the membrane to an estimated 0 mV. The data points are the average ± s.e.m. (n=4) and the figure is representative of three experiments.

increase in the time of depolarization. This may be due to greater efflux of $^{45}Ca^{2+}$ from the vesicles with longer filtration time. Also, increased mechanical pressure at longer filtration times may cause rupture of the vesicles resulting in greater loss of the trapped $^{45}Ca^{2+}$. However,the greatest amount of specific $^{45}Ca^{2+}$ flux is observed within the first 300 msec of initiation of depolarization. This is similar to the time taken for slow VDCCs to reach peak current amplitude in voltage-clamp recordings of intact skeletal muscle fibres (Beaty *et al.*, 1987). In all subsequent experiments, therefore, the $^{45}Ca^{2+}$ flux response was measured 50-100 msec after initiation of depolarization.

4.4 Effect of the Inorganic Ca^{2+} Channel Blockers on $^{45}Ca^{2+}$ Flux Response

The effects of the inorganic Ca^{2+} channel blockers, La^{3+} and Cd^{2+} on rapid (50 msec) $^{45}Ca^{2+}$ flux responses have been examined. The results are presented in figure 5. Neither Cd^{2+} nor La^{3+}, at a concentration of 1 mM, affected the amount of $^{45}Ca^{2+}$ retained by the vesicles under control conditions i.e. without change in membrane potential. If the membranes were first repolarized to an estimated -86 mV and then depolarized to an estimated 0 mV, there was a significant flux response on a 50 msec time scale. This flux response was partially inhibited by Cd^{2+} and completely inhibited by La^{3+}.

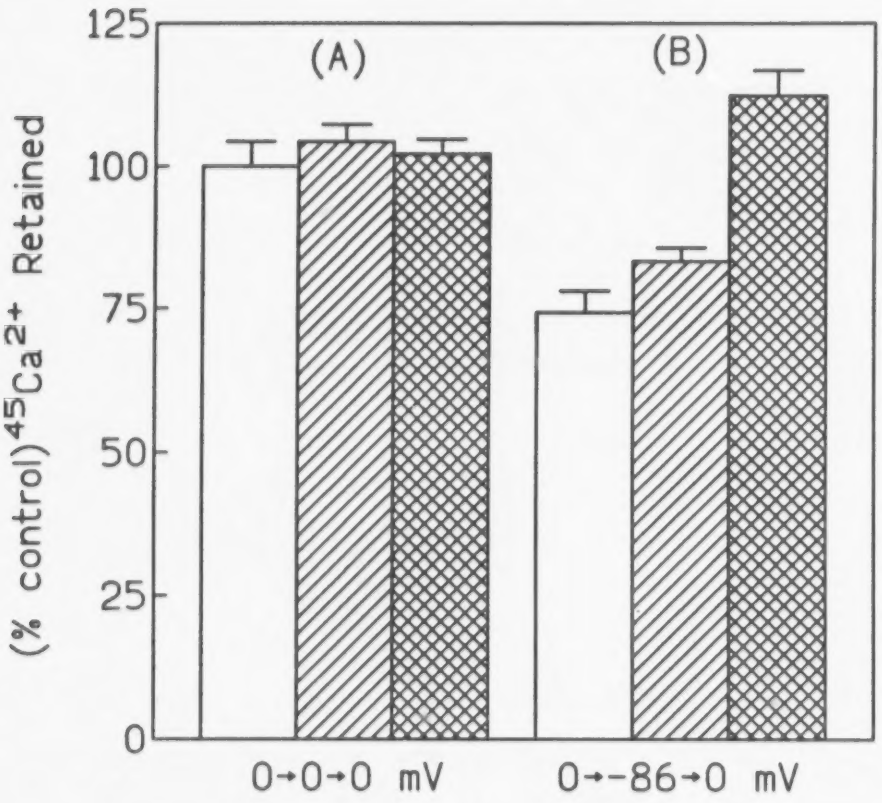


Figure 5. Effects of inorganic Ca^{2+} channel blockers on $^{45}Ca^{2+}$ flux response. T-tubule vesicles loaded with $^{45}Ca^{2+}$ were either maintained in low K^+ buffer (0 mV) (A), or were repolarized to an estimated -86 mV (B) before depolarization for 50 msec. Open bars: control; hatched bars: Cd^{2+} 1 mM; cross-hatched bars: La^{3+} 1 mM. Bars indicate average ± s.e.m. (n=4).

4.5 Effects of the DHP Ca^{2+} Channel Agonist, (±)Bay K8644, on $^{45}Ca^{2+}$ Flux Response

It has been reported earlier (Dunn, 1989) that the DHP agonist, Bay K8644 can stimulate $^{45}Ca^{2+}$ flux response both under control conditions and after depolarization from a repolarized state. Similar observations were also made with $^{45}Ca^{2+}$ flux measured at 50 msec. The effects of (±)Bay K8644 on $^{45}Ca^{2+}$ flux response are shown in figure 6. At a concentration of 0.1 μM, (±)Bay K8644 stimulated depolarization-induced $^{45}Ca^{2+}$ efflux and also reduced the amount of $^{45}Ca^{2+}$ retained by the vesicles under control conditions.

4.6 Effect of Extravesicular Ca^{2+} on $^{45}Ca^{2+}$ Flux Response

In $^{45}Ca^{2+}$ flux measurements, the extravesicular Ca^{2+} is chelated to approximately 0.1 μM by adding EGTA in the diluting buffer. This mimics the normal resting intracellular free Ca^{2+} concentration. Since one mechanism for inactivation of VDCCs in skeletal muscle is the rise in intracellular Ca^{2+}, it was of interest to investigate the effects of chelation of extravesicular (corresponding to intracellular) Ca^{2+} on the flux response (Hagiwara and Byerly, 1981; Schilling and Lindenmayer, 1984). Extravesicular Ca^{2+} was chelated to 0.01 μM - 5 mM by the use of appropriate extravesicular Ca^{2+}/EGTA solutions and the effects of this on depolarization-induced $^{45}Ca^{2+}$ flux was examined. The results of such an experiment

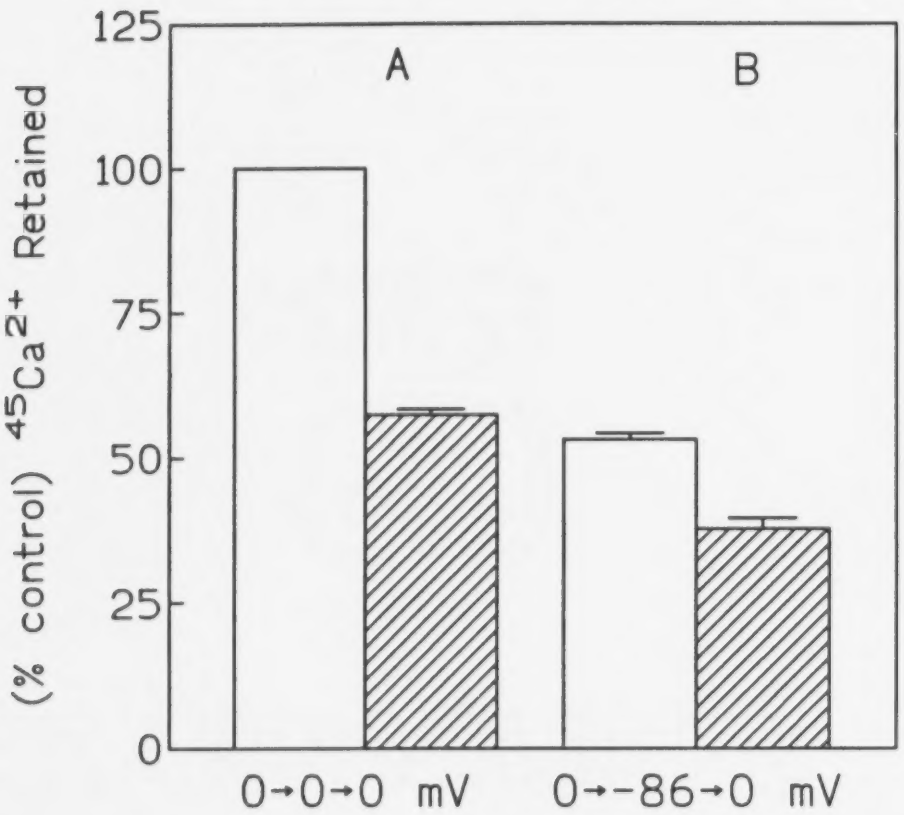


Figure 6. Effect of DHP agonist (±)Bay K8644 on $^{45}Ca^{2+}$ flux response. $^{45}Ca^{2+}$ loaded vesicles were either maintained in a depolarized state (A) or were repolarized to an estimated -86 mV before subsequent depolarization to 0 mV for 50 msec (B). The vesicles were preincubated with the drug for 30 min. Open bars: control; Hatched bars: Bay K8644 0.1 μM. Bars indicate average ± s.e.m. (n=4).

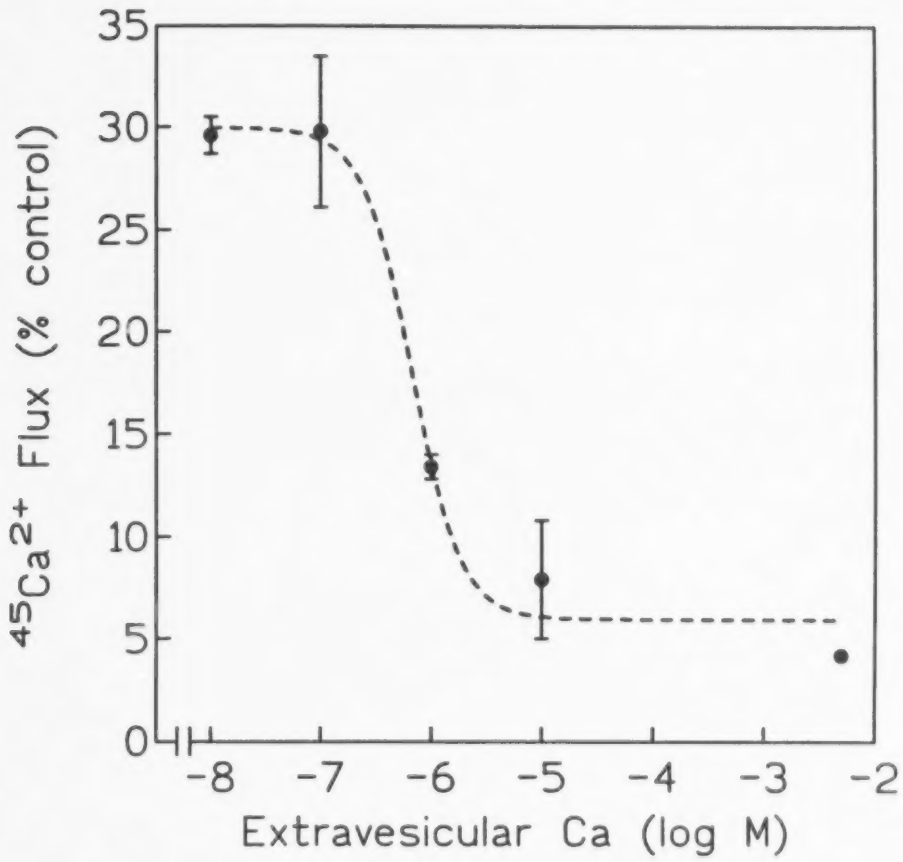


Figure 7. Effect of extravesicular Ca^{2+} on the $^{45}Ca^{2+}$ flux response. T-tubule vesicles loaded with 5 mM $^{45}Ca^{2+}$ were repolarized to an estimated -86 mV by diluting in buffers containing the desired concentrations of EGTA to chelate extravesicular Ca^{2+} to the indicated concentrations. The flux response was initiated by depolarizing the vesicles to an estimated 0 mV. Flux measured under non-repolarizing conditions at 5 mM extravesicular Ca^{2+} in the presence of 1 mM La^{3+} served as control. The data points are the average ± s.e.m. from three experiments (n= 2-3).

are shown in figure 7.

Maximum depolarization-induced $^{45}Ca^{2+}$ flux was observed when the extravesicular Ca^{2+} was chelated to about 0.1 μM. The flux was minimal at an extravesicular Ca^{2+} concentration of 10 μM, which is in the range of intracellular Ca^{2+} concentration achieved when the cell is activated. Thus, the flux is inhibited by extravesicular (intracellular) Ca^{2+} with an IC_{50} of 0.7 μM. This is similar to the concentrations of Ca^{2+} required to activate 50% maximal force in skinned skeletal muscle fibres (Thomas, 1982; Rüegg, 1986).

4.7 Voltage-Dependent Removal of Inactivation of Ca^{2+} Channels and the Effect of the Ca^{2+} Channel Antagonist, Nifedipine

Voltage-dependent Ca^{2+} channels in isolated membrane preparations seem to be inactivated, which is likely to be due to the sustained depolarization that occurs during membrane preparation (Dunn, 1989). The inactivation can however be removed by repolarization of the membrane. This property of VDCCs in transverse tubule membranes has been studied by investigating the dependence of the flux response on the membrane potential of reactivation. In these experiments, membrane vesicles loaded with $^{45}Ca^{2+}$ in low K^+ (5 mM) buffer, were repolarized to different reactivation potentials by diluting into buffers containing different potassium concentrations. After 3 min repolarization the flux was initiated by depolarization to

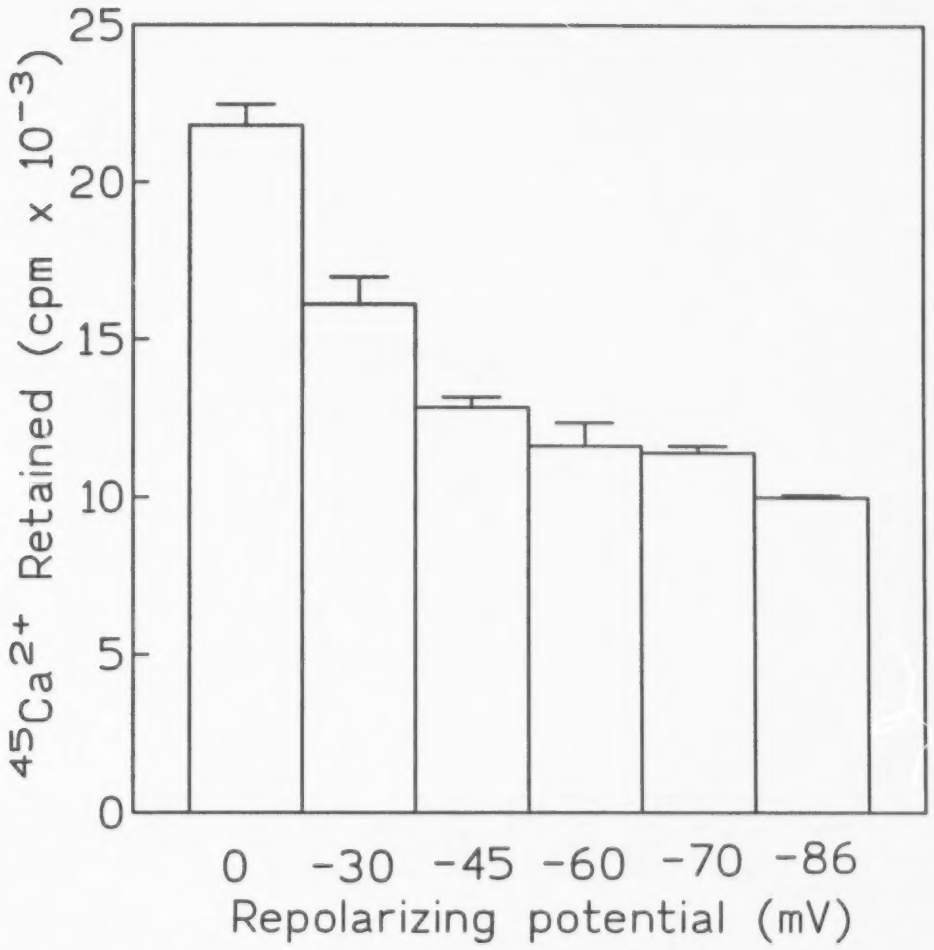


Figure 8. Dependence of $^{45}Ca^{2+}$ flux response on voltage-dependent reactivation of Ca^{2+} channels in t-tubule vesicles. Vesicles loaded with $^{45}Ca^{2+}$ in low K^+ buffer were repolarized to the indicated potentials by dilution into buffers containing appropriate K^+ concentrations. At the end of 3 min. incubation, the flux was initiated by depolarization of the vesicles for 50 msec. Data points are average ± s.e.m. (n= 9-12).

approximately 0 mV for 50 msec. As shown in figure 8, an increased flux is seen as the reactivation potential during membrane repolarization is made more negative.

1,4-Dihydropyridine antagonists bind with high affinity to cardiac VDCCs that are in an inactivated state (i.e. at depolarized potential) and their pharmacological effects are prominent only under depolarized conditions (Hosey and Lazdunski, 1988). Similar voltage-dependence of nifedipine effects have been seen in skeletal muscle t-tubule membranes (figure 9). At relatively positive repolarizing potentials (e.g. -30 mV), nifedipine (10 μM) showed a greater inhibitory effect (~ 96% inhibition) on the $^{45}Ca^{2+}$ efflux initiated by subsequent depolarization. This inhibitory effect was less marked (~46% inhibition) when a more negative repolarizing potential (e.g. -86 mV) was used. Thus, nifedipine appears to have a voltage-dependent effect on the $^{45}Ca^{2+}$ flux through VDCCs in the transverse tubule vesicles.

4.8 Effect of Extravesicular Proteolysis on $^{45}Ca^{2+}$ Flux Response

When skeletal muscle Ca^{2+} channels were expressed in murine cell lines, it was demonstrated that intracellular trypsin treatment enhanced the voltage-dependent Ca^{2+} current amplitude, and altered the inactivation and drug sensitivity of the channels (Lory *et al.*, 1992). This change in the channel properties was attributed to a selective proteolysis of the β subunit of the Ca^{2+} channel protein. However, no direct evidence was presented. The inside-out orientation of the isolated

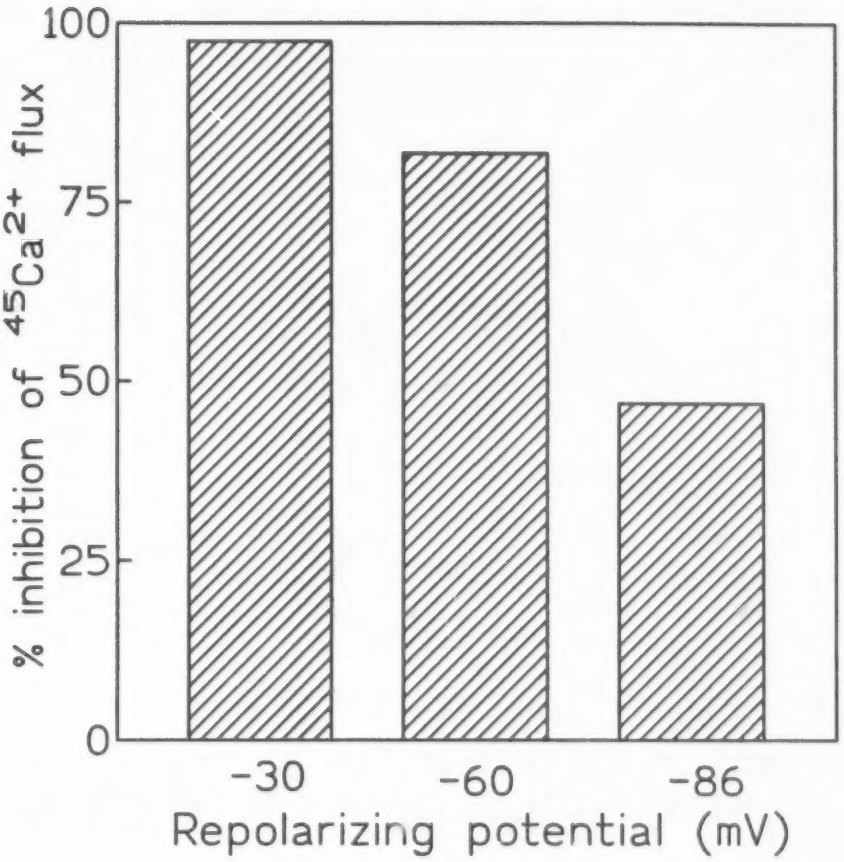


Figure 9. Effect of nifedipine (10 μM) on the voltage-dependent $^{45}Ca^{2+}$ flux response. Vesicles loaded with $^{45}Ca^{2+}$ were preincubated for 30 min with 10 μM nifedipine before repolarizing to the indicated potential for 3 min and subsequent depolarization to initiate the flux response. All buffers contained the same concentration of nifedipine. The inhibition of flux initiated from each repolarizing potential in the presence of 1 mM La^{3+} was considered 100% inhibition.

transverse tubule vesicles provides an ideal model to examine the effect of intracellular proteolysis on voltage-dependent $^{45}Ca^{2+}$ flux responses since intracellular proteins are exposed extravesicularly. The effect of trypsin treatment on depolarization-induced $^{45}Ca^{2+}$ flux responses (figure 10) was investigated . Vesicles loaded with $^{45}Ca^{2+}$ in low K^+ buffer were repolarized for 3 min in high K^+ buffer containing trypsin (1-100 μg/ml) before initiation of the depolarization-induced flux response. Trypsin(1-50 μg/ml) showed a concentration-dependent stimulatory effect on the $^{45}Ca^{2+}$ flux response with a maximum 15% increase seen at 50 μg/ml.

4.9 Effect of Membrane Potential on High Affinity Binding of DHP Ligands to Transverse Tubule Membranes

In skeletal muscle, including cardiac muscle and neuronal tissues, the pharmacological effects of DHP drugs are seen at concentrations 2-3 orders of magnitude greater than those required for the high affinity binding of radiolabelled ligands to isolated membranes (Triggle and Rampe, 1989). This may be explained by the ability of these ligands to bind with higher affinity to the depolarized state of the channel protein as occurs in isolated membranes (Kamp and Miller, 1987). In electrophysiological experiments using cardiac myocytes and skeletal muscle derived cell lines, it has been demonstrated that the potency of DHP Ca^{2+} channel antagonists is increased at depolarized membrane potentials (Bean, 1984; Sanguinetti and Kass, 1984; Cognard *et al.*, 1986).

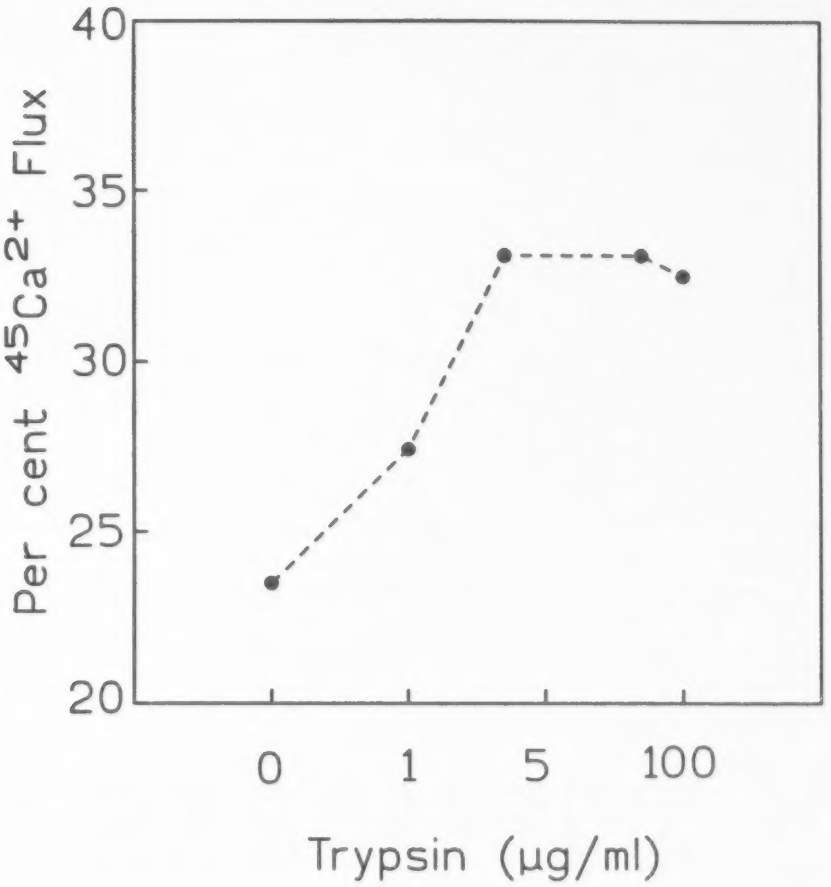


Figure 10. Effect of extravesicular trypsin on depolarization-induced $^{45}Ca^{2+}$ flux response in t-tubule membrane. T-tubule vesicles loaded with $^{45}Ca^{2+}$ were repolarized to an estimated -86 mV for 3 min by diluting in 150 mM K^+ buffer containing trypsin. The flux was initiated by depolarizing the vesicles for 100 msec. by force filtering with 5 mM K^+ buffer. The flux measured without repolarization and in the presence of 1 mM La^{3+} served as control.

In $^{45}Ca^{2+}$ flux experiments, the inhibitory effect of the DHP antagonist, nifedipine appears to be greater at depolarized membrane potential (figure 9). Hence, it is important to examine the possible effect of membrane potential on the ability of DHP ligands to bind to the channel proteins. The dependence on membrane potential of the high affinity binding of DHP ligand [^{3}H]PN200-110 was examined by studying the kinetics of association of this ligand to isolated transverse tubule membranes at 0, -30, and -86 mV. [^{3}H]PN200-110 binds to these membranes with high affinity (figure 11). However, the high affinity binding of [^{3}H]PN200-110 to isolated transverse tubule membranes does not appear to be dependent on the membrane potential (figure 12). The apparent association rate constants measured (0.289 ± 0.03 min^{-1} at 0 mV; 0.236 ± 0.02 min^{-1} at -30 mV; 0.272 ± 0.02 min^{-1} at -86 mV) were not significantly different.

4.10 Identification of Low-Affinity Binding Sites for DHP Ligand Amlodipine by Acceleration of [^{3}H]PN200-110 Dissociation Kinetics

The fact that the concentrations of DHP drugs required to affect the Ca^{2+} channel function are higher than those required to saturate the high affinity binding sites may be explained by the interaction of these ligands with distinct low affinity sites (Dunn and Bladen, 1992) and/or by the voltage-dependent binding to isolated membranes as described above. The presence of distinct low affinity binding sites for DHP ligands in the skeletal muscle transverse tubule membranes was first revealed

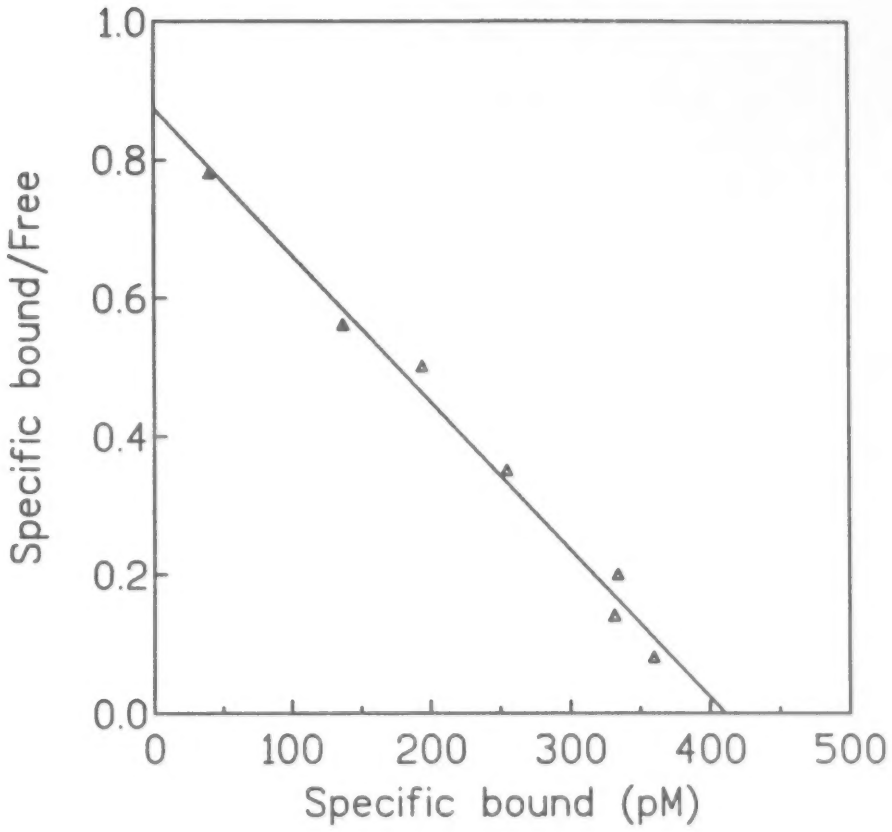


Figure 11. Representative Scatchard plot of [^{3}H]PN200-110 binding to t-tubule membranes under equilibrium conditions. Non-specific binding was estimated from parallel measurement of binding in the presence of excess of unlabelled nitrendipine. Linear least square analysis estimated K_d = 0.5 nM and B_{max} = 20.8 pmol/mg protein.

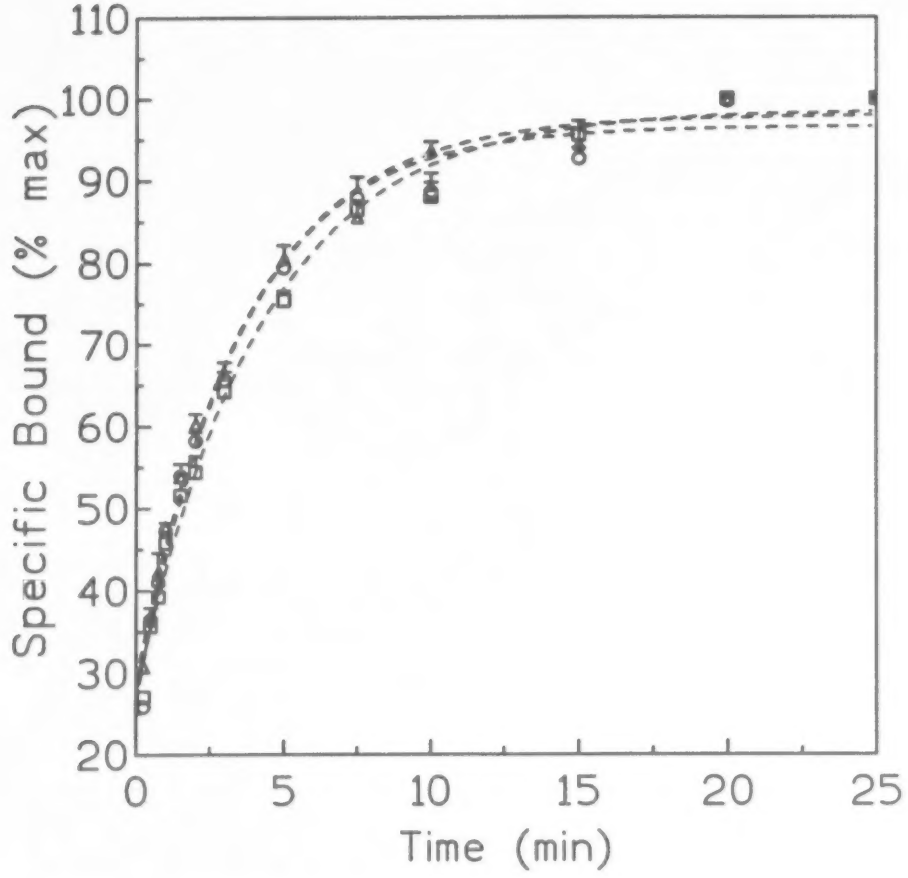


Figure 12. Effect of membrane potential on [^{3}H]PN200-110 binding to t-tubule membrane vesicles measured by association kinetics experiments. Binding was measured at indicated potentials by incubating the membranes in buffers containing appropriate K^+ concentrations. The apparent association rate constants measured are 0.289 ± 0.03 min^{-1} (0 mV, ○); 0.236 ± 0.02 min^{-1} (-30 mV, □); and 0.272 ± 0.022 min^{-1} (-86 mV, ▵). The data points are average ± s.e.m. (n=3).

by the ability of micromolar concentrations of several DHP derivatives to accelerate the rate of dissociation of [^{3}H]PN200-110 from its high affinity sites (Dunn and Bladen, 1991).

The low-affinity DHP binding sites have been further characterized using a fluorescent DHP ligand, felodipine, whose fluorescence is enhanced upon its binding to transverse tubule membranes (Dunn and Bladen, 1992). In the present study, the DHP ligand, amlodipine was used to study low-affinity binding sites in isolated transverse tubule membranes. Amlodipine has one advantage over felodipine in that it is soluble in water whereas felodipine has limited aqueous solubility. Similar to earlier observations with other 1,4-dihydropyridine ligands (Dunn and Bladen, 1991; 1992), micromolar concentrations of amlodipine accelerated the dissociation of 1 nM of [^{3}H]PN200-110 from its high affinity binding sites to which it was previously bound (figure 13). This suggests that the high- and low-affinity binding sites are allosterically linked, although it is no yet known whether they are located on the same protein complex or on separate but interacting proteins.

4.11 Fluorescence Enhancement of Amlodipine Upon its Binding to Transverse Tubule membranes

As reported earlier for felodipine (Dunn and Bladen, 1992), amlodipine (5 μM) exhibits a broad emission spectrum with maximum fluorescence occurring at 450

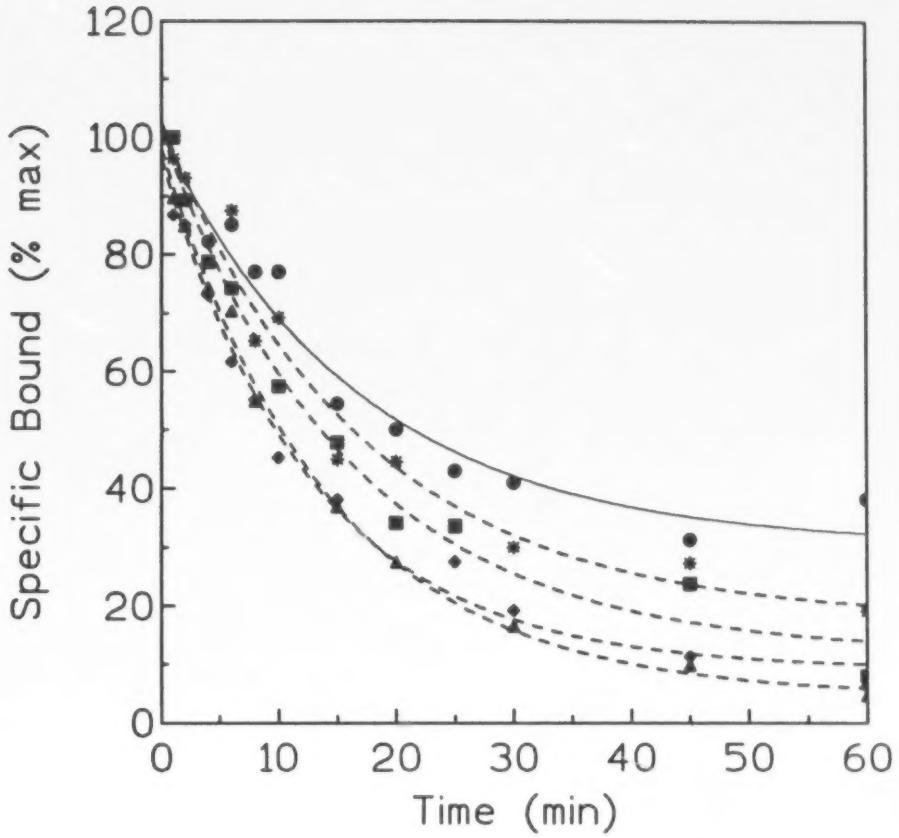


Figure 13. Effect of amlodipine on the rate of dissociation of [^{3}H]PN200-110 from high affinity binding sites in t-tubule membrane vesicles. T-tubules in 25 mM Hepes-Tris-HCl, 1 mM $CaCl_2$, pH 7.4 were incubated with 1 nM [^{3}H]PN200-110 for 45 min. The dissociation was initiated by diluting the membranes 20 fold into buffer alone (control, ●) or into buffers containing 1 μM (*), 3 μM (■), 10 μM (♦) or 30 μM (▲) amlodipine. Dissociation rate constants calculated from best fit parameters are 0.059 min^{-1} (●), 0.059 min^{-1} (*), 0.063 min^{-1} (■), 0.07 min^{-1} (♦) and 0.078 min^{-1} (▲).

nm when excited at 370 nm. This fluorescence increases by approximately 2 fold upon addition of transverse tubule membranes (5 μg/ml), and this enhancement was reversed by the addition of excess (50 μM) nitrendipine (figure 14).

The binding of amlodipine to transverse tubule membranes can also be monitored by energy transfer from transverse tubule membrane proteins (figure 15). Transverse tubule membranes are highly fluorescent presumably because of an abundance of tyrosine and tryptophan residues in the membrane proteins, and when excited at 290 nm, the fluorescence emission spectrum shows maximum at about 330 nm. In the presence of amlodipine (5 μM), the membrane protein fluorescence is quenched and a secondary emission spectrum appears with maximum fluorescence occurring between 430 and 450 nm. This results from the activation of amlodipine fluorescence by energy transfer from membrane protein. Since the energy transfer from the donor molecule to the acceptor fluorophore can occur only over a limited distance, this places restrictions on the distance of amlodipine from the protein donor (Dunn and Bladen, 1992).

The concentration-dependent fluorescence enhancement of amlodipine upon its binding to transverse tubule membranes can be measured using equilibrium fluorescence titrations, either by direct excitation or by energy transfer from membrane proteins. An example of such a titration using energy transfer is presented in figure 16. Amlodipine shows a concentration-dependent increase in its fluorescence

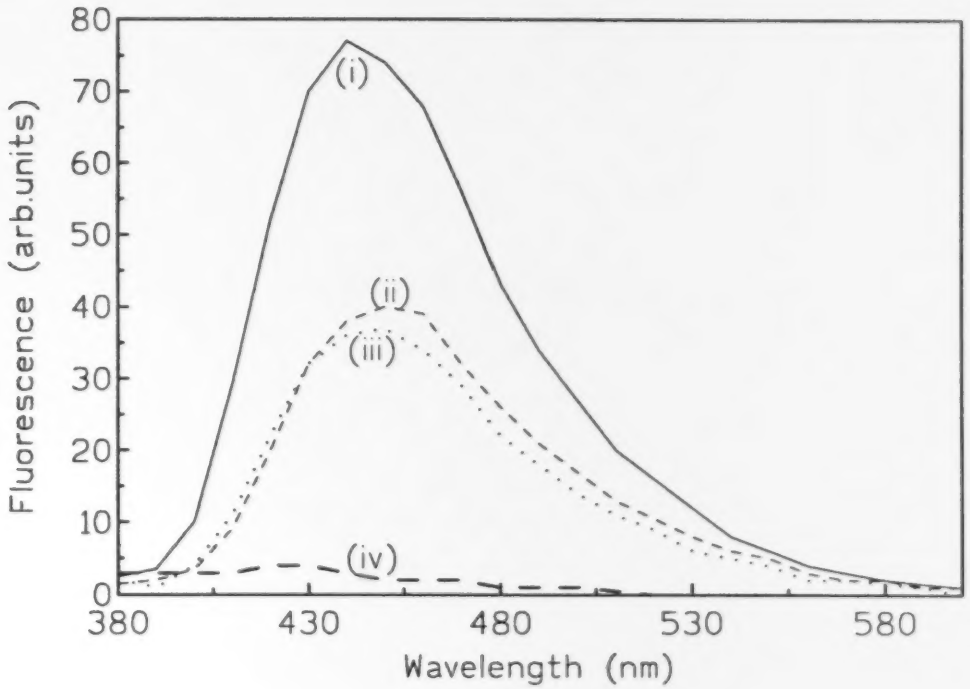


Figure 14. Enhancement of amlodipine fluorescence upon its binding to transverse tubule membranes measured by direct excitation at 370 nm. Fluorescence emission spectra of 5 μM amlodipine in 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 with (i) and without (ii) added 5 μg/ml of transverse tubules. Nitrendipine 50 μM inhibits the enhancement of amlodipine fluorescence upon binding to t-tubules (iii) whereas t-tubule alone does not emit fluorescence in the absence of amlodipine (iv).

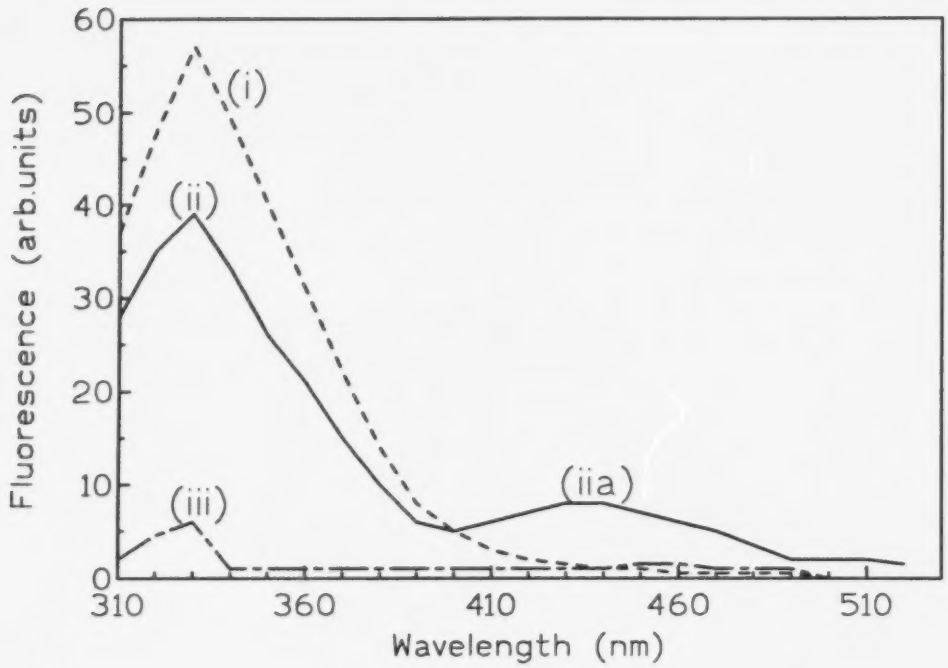


Figure 15. Excitation of amlodipine (5 μM) fluorescence via energy transfer from t-tubular protein excited at 290 nm. The protein fluorescence at 330 nm (i) gets quenched in the presence of 5 μM amlodipine (ii) with amlodipine in turn emitting fluorescence at about 450 nm (iia). Amlodipine (5 μM) cannot be excited directly at 290 nm to emit fluorescence (iii).

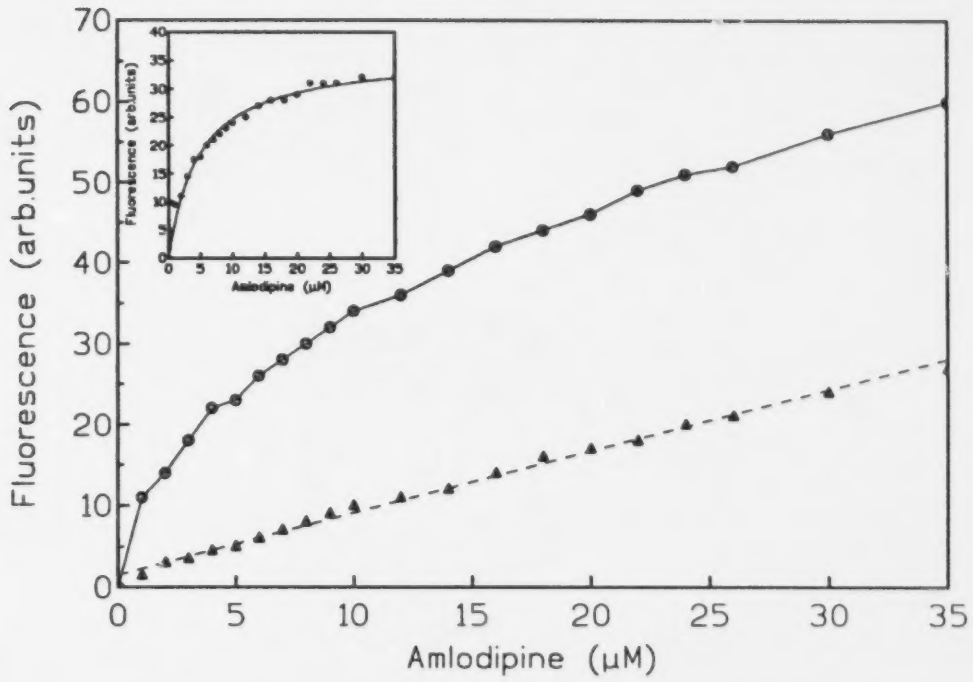


Figure 16. Fluorescence titration of transverse tubule membranes by amlodipine by energy transfer. T-tubule membranes (10 μg/ml in total of 2 ml) in 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 (o) or buffer alone (Δ) were continuously stirred and titrated with aliquots of stock amlodipine in buffer to give indicated final concentrations. The fluorescence was recorded at 450 nm using excitation wavelength of 290 nm. The inset shows the curve indicating the saturable specific enhancement of amlodipine fluorescence as a result of its binding to t-tubule membrane proteins. The apparent K_d value of amlodipine using best-fit parameters is 4.7 ± 0.31 μM.

that is not saturable. However, in the presence of added transverse tubule membranes, there is a specific saturable increase in amlodipine fluorescence giving an apparent K_d value for binding of 4.7 ± 0.31 μM. Amlodipine alone does not emit fluorescence when excited at 290 nm (figure 15). However, the non-saturable increase in its fluorescence as shown in figure 16 may be the result of its direct excitation under the experimental conditions used (e.g. wider slit width in the spectrofluorometer).

4.12 Effect of (±)Bay K8644 on Amlodipine Fluorescence

It has been reported earlier (Dunn and Bladen, 1992) that the enhancement in the fluorescence occurring as a result of the binding of ligand (felodipine) to the transverse tubule membrane can be reversed by other 1,4-dihydropyridine ligands. Similar results were obtained with the fluorescent ligand, amlodipine, as shown in figure 17. The fluorescence enhancement of amlodipine by transverse tubule membrane was inhibited by increasing concentrations of (±)Bay K8644. (±)Bay K8644 reversed the amlodipine fluorescence in a competitive manner with an apparent inhibition constant (K_i) of 3.3 μM and IC_{50} value of 5.6 ± 0.3 μM. The non-specific effect due to absorbance by Bay K8644 was corrected using parallel titrations with amlodipine in the absence of transverse tubule membranes.

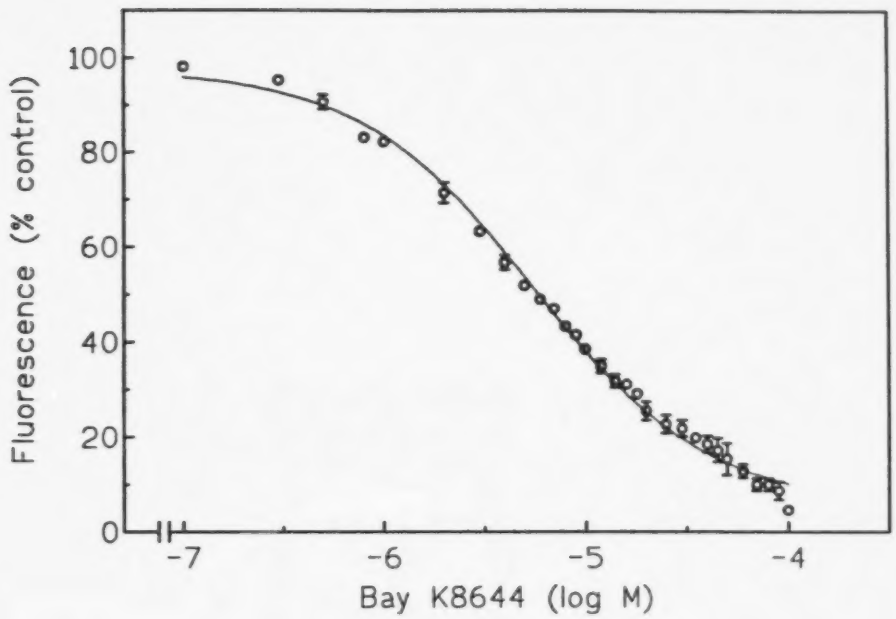


Figure 17. Effect of DHP agonist (±)Bay K8644 on the enhancement of amlodipine fluorescence. Transverse tubule membranes (10 μg/ml) were incubated with 5 μM amlodipine in 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 at 25°C for 15 min. The enhancement of fluorescence emitted at 450 nm using excitation wavelength of 370 nm was titrated with increasing concentrations of (±)Bay K8644 added cumulatively from stock solution in DMSO. The non-specific effect due to absorbance by Bay K8644 was corrected using parallel titrations with amlodipine in the absence of transverse tubules. The apparent inhibition constant (K_i) and IC_{50} of (±)Bay K8644 calculated using best-fit parameters are 3.3 μM and 5.6 ± 0.3 μM, respectively. The data are average ± s.e.m. (n=3).

5. DISCUSSION

5.1 Introduction

The major finding of this study is that functional properties of voltage-dependent Ca^{2+} channels (VDCCs) can be studied in transverse tubule membranes isolated from rabbit skeletal muscle. Transverse tubule membranes contain a high density of proteins that bind the 1,4-dihydropyridine class of L-type Ca^{2+} channel ligands (Fosset *et al.*, 1983). This is consistent with the electrophysiological localization of VDCCs in this membrane system (Almers *et al.*, 1981). It has been suggested that only a few percent of the DHP binding proteins act as functional Ca^{2+} channels (Schwartz *et al.*, 1985). However, each of these proteins is likely to have intrinsic Ca^{2+} channel activity, although with a low open channel probability (Lamb, 1991, 1992). The functional significance of DHP binding proteins in skeletal muscle excitation-contraction (e-c) coupling is not clearly understood, although it is currently believed that they serve a dual role as both voltage-sensor and as VDCC (Ríos and Brum, 1987). Hence, it is important to study the ability of these proteins to respond to a change in the potential across the transverse tubule membrane and to act as Ca^{2+} channel molecules.

The Ca^{2+} channel activity of DHP binding proteins in transverse tubule membranes has been studied after their reconstitution into phospholipid vesicles (Curtis and Catterall, 1986) or planar lipid bilayers (Affolter and Coronado, 1985; Flockerzi *et al.*, 1986; Ma *et al.*, 1991). Although these methods have provided new

insights into the kinetic and pharmacological properties of skeletal muscle VDCCs, only a few active channels can be examined with these techniques. Study of the properties of the channel population as a whole is essential to understand the functional properties of these proteins.

Isolated transverse tubule membranes, in many respects, provide an ideal *in vitro* model to study the functional responses of VDCCs in their native membrane environment. When isolated, these membranes form sealed vesicles with an inside-out orientation (Hidalgo *et al.*, 1986; Dunn, 1989). The relationship between the properties of ligand binding to these membranes and the functional responses of the overall channel population can conveniently be studied using such isolated membrane preparations. Transverse tubule membrane vesicles are capable of generating and maintaining membrane potentials in response to potassium ion gradients established across the membrane in the presence of valinomycin. These potentials are readily reversible and dissipate slowly over a period of minutes to hours (Dunn, 1989).

Since isolated transverse tubule membrane vesicles are inside-out in orientation, the normal VDCC activity i.e. influx of extravesicular Ca^{2+} is mimicked by measuring Ca^{2+} efflux from vesicles preloaded with $^{45}Ca^{2+}$. In the present study, extravesicular Ca^{2+} concentration was reduced to approximately 0.1 μM by added EGTA to mimic the intracellular milieu of a cell (with right side-out orientation). This maneuver would also relieve the possible block of the Ca^{2+} channels by

intracellular Ca^{2+} (Hagiwara and Byrely, 1981). A second factor to be considered is that the VDCCs in isolated membranes likely remain in an inactivated state as a result of sustained depolarization (Almers *et al.*, 1981). It is, therefore, essential to remove inactivation by polarizing the membrane to an outside negative membrane potential to simulate the resting state of the cell *in vivo*. These channels can subsequently be activated by depolarization.

Based on the above rationale, a two step Ca^{2+} efflux assay developed by Dunn (1989) was used to study VDCC activity in isolated transverse tubule membrane vesicles (figure 2). As described in Methods (see section 3.2.4), in the first step of this method, the membrane vesicles loaded with $^{45}Ca^{2+}$ were diluted into a buffer designed to mimic the resting state of the cell which would reactivate the otherwise inactivated Ca^{2+} channels. In the second step, $^{45}Ca^{2+}$ efflux is initiated by depolarization of the membranes to a predetermined length of time with an appropriate buffer. The measured $^{45}Ca^{2+}$ flux presumably occurs through VDCCs since all of the specific $^{45}Ca^{2+}$ flux can be inhibited by the inorganic Ca^{2+} channel blocker, La^{3+} (figure 3). Thus, isolated transverse tubule membranes are suitable for study of VDCCs in their membrane environment.

5.2 Time Course of $^{45}Ca^{2+}$ Flux Response

The $^{45}Ca^{2+}$ flux measurements using a manual filtration technique were made

by depolarizing the membranes for 20 sec (figure 2), whereas *in vivo* the VDCCs in skeletal muscle operate at much faster time scales, their conductance reaching peak at about 200 msec (Beaty *et al.*, 1987). Hence, it is important to study the functional responses of VDCCs on similar, physiologically relevant, time scales. A rapid filtration technique (Dupont, 1984) was used, in the present study, to measure $^{45}Ca^{2+}$ flux responses in the range of 10 msec to 10 sec. A similar technique has recently been used to study the kinetics of Ca^{2+} release from the junctional sarcoplasmic reticulum of rabbit skeletal muscle (Valdivia *et al.*, 1992). Using this technique, the greatest amount of specific $^{45}Ca^{2+}$ efflux stimulated by membrane depolarization occurred within 300 msec of the initiation of depolarization (figure 4). The time course is in agreement with slow Ca^{2+} currents measured in skeletal muscle using voltage-clamp techniques (Beaty *et al.*, 1987).

The VDCC activity of DHP binding proteins in transverse tubule membranes was further characterized by measuring the $^{45}Ca^{2+}$ efflux response on physiological time scales, by depolarizing the membranes for only 50-100 msec.

5.3 Effect of the Inorganic Cations on $^{45}Ca^{2+}$ Flux Response

Suppression of the Ca^{2+} current by various inorganic cations has been demonstrated in skeletal muscle fibres (Donaldson and Beam, 1983; Palade and Almers, 1985; Cota and Stefani, 1986). In line with these observations, the inorganic

Ca^{2+} channel blockers, La^{3+} and Cd^{2+}, inhibited the depolarization-induced $^{45}Ca^{2+}$ flux response measured at 50 msec in transverse tubule vesicles. At equimolar concentrations (1 mM), La^{3+} completely inhibited the flux response, whereas a partial block of the flux response was observed with Cd^{2+} (figure 5). Similar observations have been made in barnacle skeletal muscle (Hagiwara, 1973, 1975), cardiac muscle (Campbell *et al.*, 1988c) and smooth muscle (Anderson *et al.*, 1971). Valency of the blocking cation has been suggested to be directly related to its effectiveness as a competitive inhibitor of the Ca^{2+} channel (Lakshminarayanaiah, 1991).

5.4 Effect of the DHP Agonist, (±)Bay K8644, on $^{45}Ca^{2+}$ Flux Response

Ca^{2+} channel activators such as Bay K8644 and (+)-SDZ 202-791 have played an important role in defining L-type VDCCs. The activator response of the channels in the presence of these drugs is an important test for the presence of L-type Ca^{2+} channels (Janis *et al.*, 1987; Bechem *et al.*, 1988). (±)Bay K8644, at 0.1 μM, stimulated depolarization-induced $^{45}Ca^{2+}$ efflux from transverse tubule membranes. The potentiating effect of (±)Bay K8644 on $^{45}Ca^{2+}$ efflux was also seen under control conditions, i.e. in membranes that were not repolarized prior to subsequent depolarization (figure 6). The stimulatory effect of (±)Bay K8644 on the $^{45}Ca^{2+}$ flux response under control conditions may be attributed to its ability to bind to the open state of the channel and prolong its opening time under sustained depolarization

(Bechem and Schramm, 1988). Bay K8644 has been shown to act as an activator of Ca^{2+} channel in mammalian skeletal muscle cells in culture (Cognard *et al.*, 1986).

5.5 Effect of Extravesicular Ca^{2+} on $^{45}Ca^{2+}$ Flux Response

During a maintained depolarization, the activation of Ca^{2+} current is followed by a slower decay process called inactivation. There are two well described mechanisms of Ca^{2+} channel inactivation in neurons (Chad, 1989): (i) voltage-dependent and (ii) Ca^{2+}-dependent inactivation. In the Ca^{2+}-dependent mechanism, the inactivation of Ca^{2+} channels is sensitive to cytoplasmic Ca^{2+} concentration. Thus, intracellular Ca^{2+} controls further Ca^{2+} entry via a negative feedback mechanism, and recovery from this inactivation is largely determined by internal Ca^{2+} buffering. In electrophysiological studies, replacing Ca^{2+} with Ba^{2+} as charge carrier or chelating intracellular Ca^{2+} with either EGTA or BAPTA has been shown to remove inactivation of the Ca^{2+} channel, whereas increasing intracellular Ca^{2+} increases Ca^{2+} channel inactivation in neurones and cardiac muscle (Chad, 1989).

In skeletal muscle, the slow inactivation of the L-type VDCC current observed during depolarization of the muscle fibres has been attributed to a time-dependent depletion of Ca^{2+} in the transverse tubule rather than a Ca^{2+}-dependent mechanism (Stanfield, 1977; Almers *et al.*, 1981; Palade and Almers, 1985; Beam and Knudson, 1988). The possibility of Ca^{2+}-dependent inactivation can be directly studied in

isolated transverse tubule membrane vesicles. In membrane vesicles loaded with $^{45}Ca^{2+}$, the extravesicular Ca^{2+} (that *in vivo*, represents the intracellular Ca^{2+}) can be buffered to the desired concentration by adding Ca^{2+} chelating agents such as EGTA to the repolarizing buffer. Subsequently, the dependence of depolarization-induced $^{45}Ca^{2+}$ efflux on extravesicular Ca^{2+} concentration can be examined. The $^{45}Ca^{2+}$ efflux response was maximum when extravesicular Ca^{2+} was chelated to approximately 0.1 μM and the response reached its minimum at an extravesicular Ca^{2+} concentration of 10 μM. Thus Ca^{2+} flux was maximum under conditions in which extravesicular Ca^{2+} was buffered to free concentrations similar to those occurring under resting cellular conditions and a minimum flux response was observed in the range of intracellular Ca^{2+} that is reached when the cell is activated (10 μM) (figure 7; Rüegg, 1986). This is suggestive of the presence of a Ca^{2+}-dependent inactivation process (Cognard *et al.*, 1986). A Ca^{2+}-dependent inactivation has been demonstrated in insect skeletal muscle (Ashcroft and Stanfield, 1982).

In addition to Ca^{2+}-dependent inactivation, recovery of Ca^{2+} channels from inactivation has also been shown to be dependent on the free intracellular Ca^{2+} concentration (Yatani *et al.*, 1983; Gutnick *et al.*, 1989). The ability of hyperpolarizing pulses to restore inactivated Ca^{2+} currents is enhanced when the intracellular Ca^{2+} is low. The effectiveness of the hyperpolarizing pulse to remove inactivation is maximal when intracellular Ca^{2+} is less than 0.1 μM. A similar mechanism may be responsible for the results observed in transverse tubule membranes (figure 7). Thus

at 0.1 μM extravesicular Ca^{2+}, the repolarizing buffer is able to remove inactivation of the channel leading to a maximum flux response upon subsequent depolarization. It is unlikely that the maximum flux measured at 0.1 μM extravesicular Ca^{2+} is due to a greater Ca^{2+} ion gradient across the transverse tubule membrane since this gradient is substantial even when the extravesicular Ca^{2+} is 10 μM, and there is minimal flux.

5.6 Membrane Potential Dependent Reactivation of VDCCs

A more classical voltage-dependent inactivation of VDCCs has been shown to be present in skeletal muscle (Sanchez and Stefani, 1983; Cota *et al.*, 1984; Cognard *et al.*, 1986; Beam and Knudson, 1988). Thus, a conditioning prepulse to depolarizing potentials that do not activate any measurable Ca^{2+} current produces significant inactivation of Ca^{2+} current. Conversely, hyperpolarizing prepulses enhance recovery of channels from inactivation. As in these previous observations, the presence of voltage-dependent inactivation of VDCCs was also observed in isolated transverse tubule membrane vesicles (figure 8). The dependence of depolarization-induced $^{45}Ca^{2+}$ flux on the repolarizing membrane potential was examined. The channels remain inactivated in isolated membranes, most likely as a result of sustained depolarization occurring during membrane isolation. Increased flux was measured when the repolarizing potential was more negative prior to depolarization to the same (0 mV) potential. Inactivation of a greater proportion of

the channels will be relieved at more negative repolarizing potentials and thus more channels are available for subsequent activation.

5.7 Voltage-Dependent Blocking Effect of DHP Antagonist, Nifedipine on the $^{45}Ca^{2+}$ Flux Response

Although high affinity binding sites for 1,4-dihydropyridines have been identified in many excitable tissues including cardiac, smooth and skeletal muscle (Bellemann *et al.*, 1981; Bolger *et al.*, 1982; Fosset *et al.*, 1983), their functional significance has not been clearly understood. This is due to the large discrepancy between the apparent (nanomolar) affinity of the binding sites and the (micromolar) drug concentrations required for a pharmacological effect on Ca^{2+} channel function (Janis *et al.*, 1984). This discrepancy can be partially explained by voltage-dependent conformational changes of the Ca^{2+} channel protein, as well as preferential binding of the channel blockers to the inactivated closed conformation of the channel (Bean, 1984; Sanguinetti and Kass, 1984; Cognard *et al.*, 1986; Kamp and Miller, 1987). Furthermore, the channel blocking effect of dihydropyridines increases with holding membrane potentials less negative than the normal resting potentials (Sanguinetti and Kass, 1984; Cognard *et al.*, 1986). Similar results were obtained, when the effects of the DHP antagonist, nifedipine, were examined on the $^{45}Ca^{2+}$ flux response in isolated transverse tubule membranes (figure 9). Nifedipine, at 10 μM, exhibited a greater inhibitory effect on $^{45}Ca^{2+}$ efflux elicited by depolarization to 0 mV from a

repolarizing potential of -30 mV than from a repolarizing potential of -86 mV. Thus, nifedipine appears to have a voltage-dependent blocking effect on VDCC activity in the transverse tubule membranes. Thus, the results of the present study are consistent with the observations made in an earlier study where micromolar concentrations of DHP ligands were required for their pharmacological effect on the Ca^{2+} channel function in skeletal muscle (Ohkusa *et al.*, 1991). An attempt to examine the concentration-dependent effect of nifedipine on the $^{45}Ca^{2+}$ flux response was not successful. This may be due to a greater blocking effect of nifedipine on relatively small $^{45}Ca^{2+}$ flux elicited from less negative repolarizing potentials (see above).

5.8 Effect of Membrane Potential on DHP Binding to Transverse Tubule Membranes

While the concentrations of DHP drugs required for pharmacological effect on Ca^{2+} channel function lie in the micromolar range (see above), isolated skeletal muscle membranes carry sites that bind the 1,4-DHP ligands with high affinity (figure 11). In view of the suggestion that DHP drugs bind to depolarized state of the channel protein in cardiac muscle (Sanguinetti and Kass, 1984), the dependence of DHP ligand binding on the membrane potential of isolated transverse tubule membrane vesicles was investigated (figure 12). The high affinity binding of the DHP ligand [^{3}H]PN 200-110 to isolated transverse tubule membrane was found to be independent of membrane potential. Thus, the discrepancy described above between

the ligand binding data (Fosset *et al.*, 1983; Dunn, 1989) and pharmacological data (charge movement: Ríos and Brum, 1987; contractures: Eisenberg *et al.*, 1983; McCleskey, 1985; Ca^{2+} flux: Dunn, 1989) in skeletal muscle can not be explained by the voltage sensitivity of DHP binding. An alternative explanation for the quantitative discrepancies observed in the present and other studies is that the DHP binding proteins carry distinct multiple binding sites (Dunn and Bladen, 1992). Thus, the effect of DHPs on Ca^{2+} channel function may be due to their binding to distinct low affinity sites for DHPs in skeletal muscle transverse tubule membranes (Dunn and Bladen, 1991, 1992). Similar distinct binding sites for DHP agonists and antagonists have been suggested to be present in other tissues (Triggle and Rampe, 1989).

5.9 Identification of Low Affinity DHP Binding Sites on Transverse Tubule Membrane with Fluorescent DHP Ligand, Amlodipine

The presence of low affinity binding sites for DHP drugs in skeletal muscle membranes was revealed by the ability of micromolar concentrations of several DHP derivatives to accelerate the rate of dissociation of [^{3}H]PN200-110 from its high affinity binding sites (Dunn and Bladen, 1991). Similar observations were made in the present study with amlodipine (figure 13). At concentrations from 1 μM to 30 μM, amlodipine accelerated the rate of dissociation of 1 nM [^{3}H]PN200-110 from its binding sites. In these experiments, the dissociation was initiated by 20 fold dilution of the preformed membrane-ligand complex into buffer alone. Since this is not an

infinite dilution, it is possible that the ligand would rebind to its sites after dissociation, and in the presence of excess amlodipine this rebinding would be prevented thus increasing the apparent dissociation rate of [^{3}H]PN200-110. However, the concentrations of unlabelled DHP required to prevent the rebinding by saturating the high affinity sites has been shown to lie in the nanomolar range (Dunn and Bladen, 1992). At concentrations greater than 1 μM, DHPs bind to distinct low affinity sites that are allosterically linked to the high affinity sites since their occupancy of these low affinity sites greatly accelerates the rate of dissociation of [^{3}H]PN200-110 (Dunn and Bladen, 1992). Occupancy of the low affinity sites by DHPs may induce a conformational change in the DHP binding protein, thus displacing the ligand from its high affinity sites.

The low affinity DHP binding sites in the transverse tubule membranes have been characterized with a fluorescent DHP ligand, felodipine (Dunn and Bladen, 1992). At micromolar concentrations, the binding of felodipine to transverse tubule membranes results in a large saturable increase in fluorescence, with an apparent dissociation constant of approximately 6 μM. In the present study, another fluorescent DHP derivative, amlodipine, has been used to examine the presence of low affinity binding sites in the transverse tubule membranes. While felodipine used in the previous study (Dunn and Bladen, 1992) has limited aqueous solubility, amlodipine has an advantage of being water soluble, and hence higher concentrations could be used in the fluorescence experiments.

The fluorescence properties of amlodipine are similar to those of felodipine. The binding properties of amlodipine to transverse tubule membranes can be studied by measuring its fluorescence either by direct excitation or via energy transfer from membrane protein (figure 14 and 15). The fluorescence of amlodipine excited at 370 nm increases by approximately two fold in the presence of transverse tubule membrane and this increase in fluorescence can be specifically reversed by addition of excess DHP derivatives such as nitrendipine. This suggests that the increase in fluorescence results from binding of ligand to sites that specifically interact with DHP ligands. Amlodipine fluorescence can also be excited by energy transfer from transverse tubule membrane proteins. When excited at 290 nm, transverse tubular membrane proteins emit fluorescence at 330 nm. This fluorescence is primarily due to the aromatic amino acid residues, tyrosine and tryptophan present in the membrane protein. In the presence of amlodipine, fluorescence at 330 nm is quenched but energy transfer from the membrane excites the acceptor fluorophore which in turn emits fluorescence at 450 nm (figure 15). In order for the energy transfer to occur from the donor membrane protein to the acceptor fluorophore, the two molecules have to be located in a spatially restricted environment. This further suggests that increase in the fluorescence of amlodipine results from its interaction with the DHP-sensitive protein molecule and is not due to non-specific partitioning into the lipid environment of the membrane. Like felodipine, the increase in the amlodipine fluorescence resulting from its interaction with the transverse tubule membrane is saturable with an apparent dissociation constant of 5 μM (figure 16).

The finding that other DHP derivatives such as (±)Bay K8644 inhibit amlodipine fluorescence resulting from its interaction with the transverse tubule membrane (figure 17) suggests that the low affinity binding sites are located on or near the protein that binds DHP ligands. Two observations made with felodipine in the previous study further support this suggestion. There was a direct correlation between the magnitude of felodipine fluorescence enhancement and the density of high affinity DHP binding sites in the transverse tubule membranes (Dunn and Bladen, 1992). Furthermore, the two classes of binding sites are likely to be conformationally linked since there was an excellent agreement between the dose dependence of fluorescence enhancement and the ability of felodipine to accelerate the rate of dissociation of [^{3}H]PN200-110 from its high affinity binding sites (Dunn and Bladen, 1992).

Demonstration of low affinity binding of DHP ligands to purified DHP binding proteins will help to clarify whether the two binding sites are located on the same protein complex or whether two separate but conformationally linked proteins are involved. The fact that the low affinity DHP binding to transverse tubule membranes is resistant and the high affinity binding is sensitive to heat- and protease treatment (Dunn and Bladen, 1992) is suggestive that the low affinity sites are located in a protected intramembranous region. The membrane bilayer pathway theory of DHP ligand-receptor interaction proposed by Rhodes *et al* (1985) further supports this idea. Thus, the membrane lipid environment may be required for low

affinity binding of DHP ligands and this may explain the failure to identify these sites in the purified DHP binding protein preparations used by Dunn and Bladen (1992).

5.10 Effect of Extravesicular Proteolysis on $^{45}Ca^{2+}$ Flux Response

In a recent study, Lory *et al* (1992) observed that intracellular application of protease enzymes such as trypsin and carboxypeptidase increases the amplitude of Ca^{2+} current carried by the skeletal muscle α_1 subunit expressed in the murine cell line. Furthermore, intracellular proteolysis modified the channel inactivation kinetics and sensitivity of the Ca^{2+} current to DHP agonist, Bay K8644. This was attributed to the proteolytic degradation of the β subunit since the effects of proteolysis was observed only when the β subunit was co-expressed with the α_1 subunit (Lory *et al.*, 1992). While co-expression of the β subunit with the α_1 subunit was found to increase the density of high affinity DHP binding sites by about 10 fold (Varadi *et al.*, 1991), the effect of proteolysis on high affinity binding of DHP in the same preparation was not examined (Lory *et al.*, 1992). Intracellular proteolysis has been shown to modify the Ca^{2+} channel activity in other tissues including cardiac and smooth muscles (Hescheler and Trautwein, 1988; Klöckner, 1988).

In preliminary experiments it was found that extravesicular (i.e. intracellular *in vivo*) proteolysis with trypsin increases the depolarization-induced $^{45}Ca^{2+}$ flux response (figure 10). The mechanism by which trypsin enhances the Ca^{2+} current

(Lory *et al.*, 1992) or the $^{45}Ca^{2+}$ flux response is not clear. Both the α_1 and the β subunits of the DHP binding protein complex have been shown to carry specific trypsin recognition sites, and it has been suggested that intracellular proteolysis affects one or more auxiliary subunits, presumably the β subunit, thereby modifying the channel activity of the α_1 subunit (Lory *et al.*, 1992). In analogy with the proposed "ball and chain" model for the control of inactivation of sodium channels (Bezanilla and Armstrong, 1977), the β subunit has been suggested to play a "ball" role on the intracellular "chain" of the complex, and proteolytic degradation of this β subunit disrupts the inactivation mechanism (Lory *et al.*, 1992).

It is possible that proteolysis induces some conformational change in the channel protein uncovering the drug binding sites or changing the affinity of the sites for drug. In view of the finding that the high affinity DHP binding sites are destroyed by proteolysis and heat treatment (Dunn, unpublished observations), the enhanced effect of DHP drugs seen under such conditions may be due to the participation of low affinity binding sites which are resistant to these treatments. These observations, together with the fact that the concentrations of DHP drugs used in the previous studies (Ma *et al.*, 1991; Lory *et al.*, 1992) lie in the micromolar range, suggest the possible role of low affinity DHP binding sites in the regulation of VDCC activity in skeletal muscle.

The functional significance of low affinity DHP binding sites has been

described both in cardiac and skeletal muscle (Brown *et al.*, 1986; Ohkusa *et al.*, 1991). In guinea-pig cardiac myocytes, occupancy of the low affinity sites by DHP drugs has been suggested to be responsible for their stimulatory effect on the Ca^{2+} current, whereas the high affinity sites are responsible for either stimulatory or inhibitory effects depending on the membrane potential (Brown *et al.*, 1986). In skeletal muscle, modulation by DHP drugs of transverse tubule- mediated Ca^{2+} release from sarcoplasmic reticulum has been reported to occur through low affinity DHP binding sites (Ohkusa *et al.*, 1991).

Thus, the results obtained from experiments in the present study, together with the evidence presented in other studies (discussed above) suggest the presence of low affinity binding sites for DHP Ca^{2+} channel ligands in skeletal muscle transverse tubule membranes. Although the physiological significance of multiple binding sites remains to be established, it has been demonstrated that *in vivo*, the intracellular concentrations of DHP drugs can reach approximately 15 μM (Walsh *et al.*, 1988). This relatively high intracellular concentrations of drug may modulate Ca^{2+} channel function by binding to low affinity sites (Dunn and Bladen, 1992). The affinity of the binding sites may change depending on the state of the cell. For example, the DHP binding sites may change from high affinity state (measured at depolarized potentials) to low affinity state (at resting membrane potentials).

Alternatively, the existence of two distinct binding sites associated with

different functions can not be ruled out. In frog skeletal muscle, the DHP drugs, Bay K8644 and CGP-28392, at low concentrations, were found to show agonistic effect on K^+-induced contractures. However, at high (micromolar) concentrations, these drugs depress the contractile responses (Frank, 1990). This dual effect of DHP drugs may be explained by their selective binding to sites with different affinities. For example, at low concentrations, the agonistic effects are seen as a result of drug binding to the high affinity sites, whereas low affinity sites participate in the inhibitory response at high concentrations.

5.11 Conclusions

In conclusion, the results obtained in the present study suggest that the functional responses of VDCCs in skeletal muscle may be measured using isolated transverse tubule membrane vesicle preparations. Furthermore, depolarization-induced Ca^{2+} flux through VDCCs in transverse tubule membranes can readily be measured on physiologically relevant time scales using rapid filtration techniques. Comparison of the properties of DHP binding to transverse tubule membranes with their effects on the $^{45}Ca^{2+}$ flux responses suggests the presence of low affinity binding sites in addition to the well studied high affinity DHP binding sites. The low affinity binding sites can be characterized by using fluorescent DHP ligands such as amlodipine.

5.12 Future Recommendations

The results obtained from our experiments in the present study strongly suggest that functional responses of VDCCs in skeletal muscle can be measured using isolated transverse tubule membrane vesicles. The VDCC activity can be studied in the physiologically relevant time scales by using a rapid filtration technique. This technique can conveniently be used to further examine the kinetic properties of VDCCs in the transverse tubule membranes.

Although the effects of inorganic and organic Ca^{2+} channel drugs on $^{45}Ca^{2+}$ flux responses have been studied in the present and other studies (Dunn, 1989), it is necessary to characterize the detailed pharmacological properties of voltage-dependent $^{45}Ca^{2+}$ flux response in this experimental preparation. It is important to demonstrate the concentration-dependent effect of DHP drugs on the $^{45}Ca^{2+}$ flux response. This would help in establishing the relationship between the properties of ligand binding to Ca^{2+} channel population as a whole and their effect on the functional response of the Ca^{2+} channels. This is important in gaining further insight into the possible roles of VDCCs in skeletal muscle.

The low affinity DHP binding to VDCCs must be further characterized in order to understand their possible role(s) in the regulation of Ca^{2+} channels. It is necessary to examine the dependence of low affinity binding on the membrane

potential since the effect of DHPs on the Ca^{2+} channel function has been found to be voltage-dependent, whereas the high affinity DHP binding to transverse tubule membranes is independent of membrane potential. A detailed study of the kinetics of low affinity DHP binding to transverse tubule membranes may reveal further information on the binding characteristics.

Furthermore, a detailed investigation on the effect of proteolysis on (i) $^{45}Ca^{2+}$ flux response and its modulation by DHP and other drugs, and (ii) the high- and low affinity DHP binding will help in establishing a relationship between the high density of DHP binding proteins found in transverse tubule membrane system and their possible role(s) in skeletal muscle.

6. SUMMARY AND CONCLUSIONS

1. A rapid filtration technique was used to investigate the kinetics of $^{45}Ca^{2+}$ flux mediated by voltage-dependent Ca^{2+} channels (VDCCs) in transverse tubule membranes isolated from rabbit skeletal muscle. When isolated, these membranes form sealed, inside-out vesicles which can be used to measure Ca^{2+} fluxes in response to changes in membrane potential. Membrane vesicles loaded with $^{45}Ca^{2+}$ were repolarized to an estimated -86 mV by establishing potassium diffusion gradients in the presence of valinomycin. Upon subsequent depolarization, the greatest amount of specific $^{45}Ca^{2+}$ efflux was measured within 300 msec of initiation of depolarization.

2. The effect of inorganic Ca^{2+} channel blockers, La^{3+} and Cd^{2+} was tested on $^{45}Ca^{2+}$ flux response induced by depolarization of transverse tubule membranes for 50 msec. At 1 mM, La^{3+} completely inhibited depolarization-induced $^{45}Ca^{2+}$ efflux from membrane vesicles, whereas at equimolar concentrations Cd^{2+} was partially effective in inhibiting the flux response.

3. The DHP Ca^{2+} channel agonist, (±)Bay K8644 (0.1 μM) significantly increased $^{45}Ca^{2+}$ flux response induced by membrane depolarization. The potentiating effect of (±)Bay K8644 on $^{45}Ca^{2+}$ flux was also observed under control conditions, i.e. in samples that were not repolarized prior to subsequent depolarization.

4. Depolarization-induced $^{45}Ca^{2+}$ efflux from transverse tubule membrane vesicles was found to be dependent on the extravesicular Ca^{2+} concentration. The amount of $^{45}Ca^{2+}$ flux was maximum at an extravesicular Ca^{2+} concentration of 0.1 μM, and the flux was minimum when extravesicular Ca^{2+} was kept at 10 μM.

5. The Voltage-dependent nature of $^{45}Ca^{2+}$ flux response was studied by examining the dependence of flux response on the potential of channel reactivation. The amount of $^{45}Ca^{2+}$ flux measured by depolarization of the membrane was increased when the repolarizing potential was more negative. DHP antagonist, nifedipine (10 μM) was found to show voltage-dependent blocking effect on depolarization-induced $^{45}Ca^{2+}$ flux in transverse tubule membranes. The inhibitory effect of nifedipine was greater when the membranes were repolarized to a more negative (-86 mV) potentials before subsequent depolarization to 0 mV, than at less negative repolarizing potentials.

6. It was found that extravesicular proteolysis with trypsin increases depolarization-induced $^{45}Ca^{2+}$ efflux from transverse tubule membrane vesicles.

7. While the effect of DHP drugs on the functional responses of VDCCs was

found to be voltage-dependent (see above), the high affinity binding of DHP ligand [^{3}H]PN200-110 to transverse tubule membranes was independent of membrane potential.

8. The presence of low affinity DHP binding sites in transverse tubule membranes was revealed by the ability of micromolar concentrations of amlodipine to accelerate the rate of dissociation of [^{3}H]PN200-110 from its high affinity binding sites. The low affinity binding sites were further studied by measuring the fluorescence enhancement of amlodipine resulting from its binding to transverse tubule membranes. The low affinity binding of amlodipine to transverse tubule membranes was found to be saturable with an apparent dissociation constant of 5 μM, and this binding was found to be DHP-specific with DHP derivative, (±)Bay K8644 reversing the amlodipine binding in a competitive manner (IC_{50} of approximately 5 μM).

9. It is concluded that transverse tubule membrane vesicles can be used to study the functional responses of VDCCs on physiologically relevant time scales using a rapid filtration technique. Furthermore, it is concluded that DHP drugs modulate VDCC activity in a voltage-dependent manner. This channel-modulating effect of DHP drugs may involve their binding to low affinity binding sites located either on or near high affinity DHP binding sites on the channel protein complex.

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